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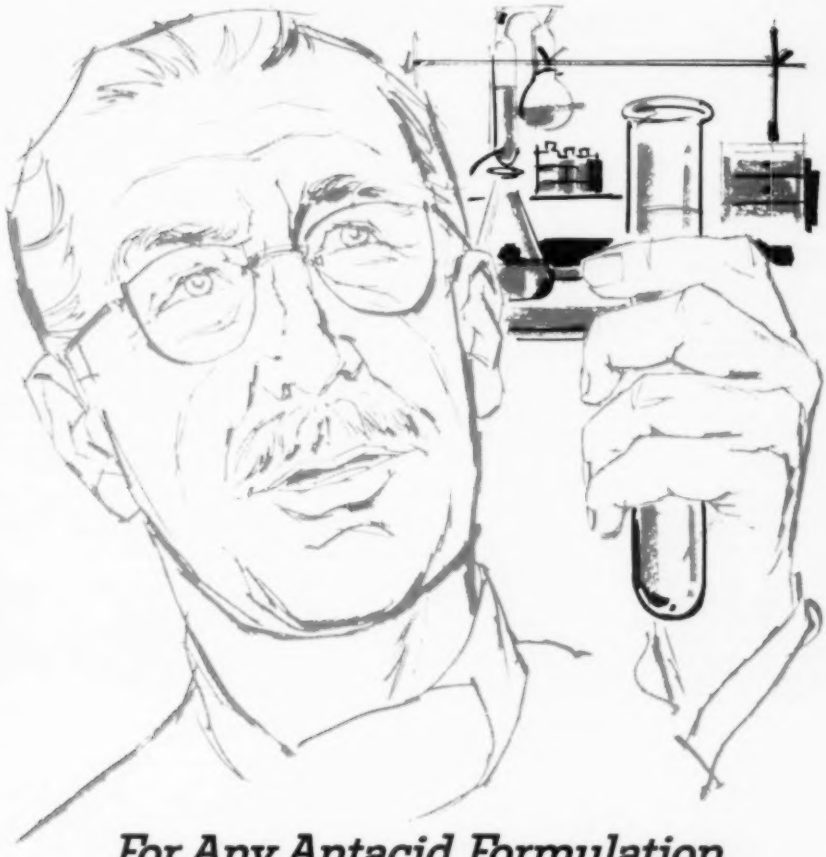

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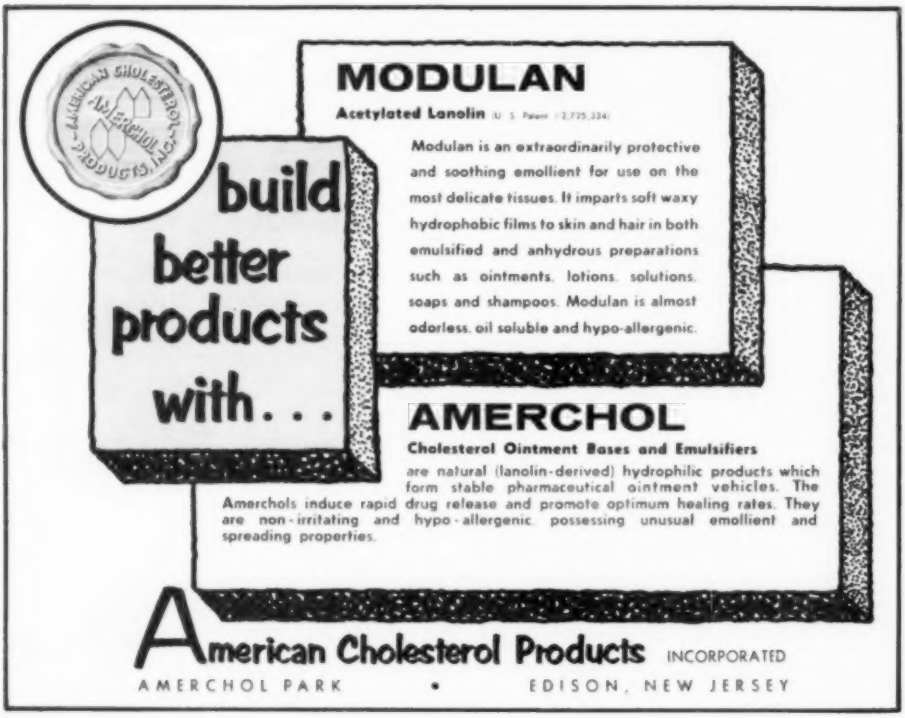
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VOLUME 49 — *FEBRUARY 1960* — NUMBER 2

Observations on Comparative Growth Studies Between a Streptomycin-Resistant and Streptomycin-Sensitive Strain of *Salmonella schottmuelleri**

By RICHARD J. SIMMONS and CHARLES GAINOR

Growth comparisons between a streptomycin-resistant and streptomycin-sensitive strain of *Salmonella schottmuelleri* is reported. The inability of the resistant strain to utilize ammonium citrate, ammonium succinate, *l*-asparagine, aspartic acid, and cystine as sole source of nitrogen stands in sharp contrast to the performance of the sensitive strain. The resistant strain failed to grow in a synthetic medium of mineral salts, glucose, and *l*-asparagine which, however, supported excellent growth of the sensitive strain. Regardless of the nature of the nutritional environments employed in this study, the resistant strain was never stimulated to a level of growth comparable to that of the sensitive strain. Apparently, the rate of utilization of glutamic acid and aspartic acid is the same for each strain, and the two strains produced alanine at approximately the same rate. In biochemical reactions the resistant strain demonstrated a response forty-eight to seventy-two hours later than the sensitive strain, and failed to produce gas from maltose or glucose in seventy-two hours. In contrast, the sensitive strain gave, in twenty-four hours, reactions comparable to a typical *S. schottmuelleri*.

THE PROBLEM of microbial resistance to antibiotics still requires additional information to explain more fully this perplexing phenomenon. Studies were undertaken to ascertain what differences, if any, exist between a streptomycin-resistant and streptomycin-sensitive strain of *Salmonella schottmuelleri* as to relative growth rates, utilization of different carbon and nitrogen sources, and general biochemical reactions.

An excellent article which reviews differences between resistant and sensitive strains of bacteria, in addition to their tolerance of diverse levels of antibiotics, has been published (1).

EXPERIMENTAL

Isolation and Maintenance of the Streptomycin-Resistant Strains.—The parent culture of *Salmonella schottmuelleri* (ATCC 9282), when subjected to the gradient plate technique (2), was completely inhibited by 10 mcg. per ml. of streptomycin in Difco nutrient agar. Resistant strains of bacteria were those that multiplied equally well in the presence or absence of high concentrations of streptomycin (5,000 mcg. per ml.). Streptomycin-resistant strains were isolated from the parent culture by a single-step selection method (3) and by serial transfers in increasing concentrations of the antibiotic (2). The resistant strain used in all of the subsequent studies could withstand 5,000 mcg. of streptomycin per ml. of medium.

Preparation of Inocula.—The preparation of inocula for all experimental studies was carefully standardized to minimize variation in the number of bacteria at the time of inoculation. Cells were carefully removed from eighteen-hour nutrient agar slants to sterile physiological saline. This suspension was centrifuged at 2,000 r. p. m. for thirty minutes, and the process repeated after adding fresh saline to the sedimented cells. The twice-washed cells were then resuspended in fresh saline, and adjusted to the desired absorbance reading. A standard curve relating absorbance readings to numbers of bacteria per ml. of suspension was pre-

* Received August 18, 1959 from the Department of Microbiology and Public Health, Michigan State University, East Lansing.

This paper is taken in part from a dissertation submitted in partial fulfillment for the degree of Master of Science, Department of Biological Sciences, Division of Bacteriology, University of Pittsburgh, Pittsburgh, Pa.

viously constructed for both the streptomycin-resistant and streptomycin-sensitive strains of *S. schottmuelleri*. Unless otherwise stated, 0.1 ml. of bacterial suspension, containing approximately 8×10^4 bacteria, was used as the inoculum per 10 ml. of medium.

Growth Comparisons in Chemically-Defined Media.—The initial growth studies employed a medium prepared as follows: A basal medium containing 0.2% Na_2SO_4, 0.002% $MgCl_2$, 0.05% KH_2PO_4, 0.15% K_2HPO_4, and 0.2% *l*-asparagine was autoclaved at 120° for ten minutes. Then, 1 ml. of a Seitz-filtered, 2% glucose solution was added aseptically to 9 ml. of basal medium, and all tubes incubated at 37° for twenty-four hours to check sterility. The pH of this medium, without adjustment, was consistently 6.9 to 7.0. It is of particular interest that the streptomycin-resistant strain would not grow in this medium, whereas the sensitive-parent grew rapidly and profusely. Experiments were then conducted to produce, if possible, a stimulation in growth of the resistant strain comparable to that of the sensitive culture by additions of supplements to the synthetic medium.

The nitrogen requirements of the two strains were investigated by replacing the *l*-asparagine of the original medium with various ammonium salts and amino acids. Final concentrations of the amino acids and ammonium salts were 0.005% and 0.1%, respectively.

A study of the carbon requirements was conducted by substituting a number of the dibasic organic acids of the Kreb's cycle for glucose, and by replacing the *l*-asparagine with cysteine-HCl in the original synthetic medium. Final concentrations of the carbon compounds and cysteine-HCl were 0.01%.

The effect of a vitamin mixture and several individual vitamins on the growth of the two strains in the original, as well as other, chemically-defined media was tested. Biotin, nicotinic acid, and pyridoxine-HCl in a final concentration of 2 mcg. per ml. of medium were used.

In critical experiments, growth was measured turbidimetrically with the Bausch and Lomb Spectronic 20 set at 425 mμ and uninoculated medium used as the reference. Otherwise, the presence or absence of growth was determined by visual inspection of inoculated tubes.

Amino Acid Utilization.—Streptomycin-resistant and streptomycin-sensitive strains of *S. schottmuelleri* were inoculated into separate 250-ml. flasks containing 100 ml. of basal medium plus 0.2% glucose, 0.005% *l*-glutamic acid, and 0.005% *l*-aspartic acid. Twice washed, eighteen hour-old cells were used in a final concentration of approximately 1×10^8 bacteria per ml. of medium. The two test systems and the uninoculated control were incubated at 37°. At various time intervals during growth the amino acid concentration of the supernatants was determined by quantitative chromatography (4). Separation of the amino acids was carried out for twenty to twenty-four hours in phenol saturated with citrate buffer (5).

Biochemical Studies.—All tests were done in triplicate. Seitz-filtered glucose, maltose, sucrose, lactose, mannitol, glycerol, inulin, xylose, cellulose, arabinose, and dextrin in a concentration of 1% in Difco nutrient broth were used. The IMVIC reactions, gelatin liquefaction, H_2S production, and activity in litmus milk were also observed.

RESULTS AND DISCUSSION

The streptomycin-sensitive strain of *S. schottmuelleri* gave rise to strains which were highly resistant to streptomycin. Colonies which appeared on plates with 5,000 mcg. per ml. of the antibiotic, when subcultured, proved to consist of highly resistant bacteria which remained so in the absence of the antibiotic. The resistant strain used throughout this study has remained essentially unchanged after repeated subculture in streptomycin-free media for four months.

The additions of a vitamin mixture and individual vitamins failed to stimulate an increase in bacterial numbers of the resistant strain regardless of the nitrogen or carbon substrate used. Actually, the two strains would grow without these vitamins and were not significantly stimulated by their presence.

The addition of streptomycin (up to 1,000 mcg. per ml.) to either nutrient broth or a chemically defined medium was without effect on the growth of the resistant strain. The resistant strain grew at approximately the same rate and to the same population levels in the presence or absence of streptomycin, thus indicating its independence of the drug.

The inability of the resistant strain to utilize ammonium citrate, ammonium succinate, *l*-asparagine, aspartic acid, cysteine-HCl, and cystine as a sole source of nitrogen, even after seventy-two hours of incubation, stands in sharp contrast to the performance of the sensitive strain (Table I).

The effect of single carbon sources on the growth of the two strains is shown in Table II.

The resistant strain grew on continued subculture in Difco nutrient broth, and in a minimal synthetic medium composed of 0.2% Na_2SO_4, 0.002%

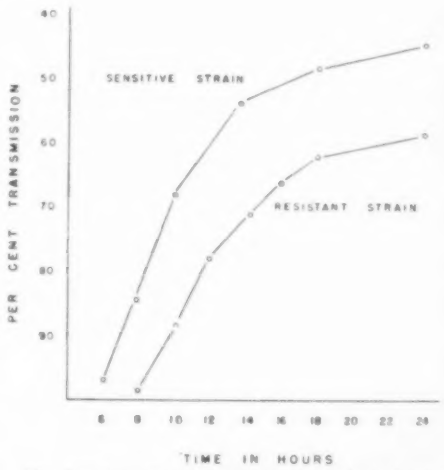

Fig. 1.—Growth curves of streptomycin-resistant and streptomycin-sensitive strains of *S. schottmuelleri* grown in Difco nutrient broth, pH 7.0, at 37°.

TABLE I.—EFFECT OF NITROGEN SOURCE ON GROWTH OF STREPTOMYCIN-RESISTANT AND STREPTOMYCIN-SENSITIVE STRAINS OF *S. schottmuelleri* IN A CHEMICALLY-DEFINED MEDIUM[a]

	Light Transmission, %							
	Sensitive, Hours				Resistant, Hours			
	18	24	48	72	18	24	48	72
Nitrogen Source Plus Glucose								
Ammonium chloride[b]	100	100	100	100	100	100	100	100
Ammonium citrate	100	100	90	80	100	100	100	100
Ammonium succinate	100	100	93	83	100	100	100	100
l-Asparagine	100	95	81	74	100	100	100	100
Aspartic acid	97	84	77	70	100	100	100	100
Glutamic acid	98	85	79	72	100	100	96	85
Cysteine-HCl	91	79	72	70	100	100	97	80
Cystine	87	74	66	60	100	100	90	76
Nitrogen Source Minus Glucose								
Ammonium chloride[b]	100	100	100	100	100	100	100	100
Ammonium citrate	100	100	95	88	100	100	100	100
Ammonium succinate	100	100	100	93	100	100	100	100
l-Asparagine	100	100	94	85	100	100	100	100
Aspartic acid	100	100	96	92	100	100	100	100
Glutamic acid	100	96	88	80	100	100	100	95
Cysteine-HCl	100	100	100	100	100	100	100	100
Cystine	100	100	100	93	100	100	100	100

[a] Medium contains 0.2% Na_2SO_4, 0.002% $MgCl_2$, 0.05% KH_2PO_4, 0.15% K_2HPO_4, and 0.2% glucose, where indicated.
[b] Same results also apply to ammonium nitrate, ammonium phosphate, and methionine. 100, no growth; 99–90, poor growth; 89–80, fair growth; 79–70, good growth; and 69–60, excellent growth.

TABLE II.—EFFECT OF CARBON SOURCE ON GROWTH OF STREPTOMYCIN-RESISTANT AND STREPTOMYCIN-SENSITIVE STRAINS OF *S. schottmuelleri* IN A CHEMICALLY-DEFINED MEDIUM[a]

	Light Transmission, %							
	Sensitive, Hours				Resistant, Hours			
Carbon Source	18	24	48	72	18	24	48	72
Citric acid	100	92	84	77	100	100	100	100
Fumaric acid	100	100	91	85	100	100	100	100
Malic acid	100	93	89	83	100	100	100	93
Oxaloacetic acid	100	95	91	87	100	100	100	92
α-Ketoglutaric acid	100	97	94	92	100	100	100	98
Glucose	92	83	79	73	100	100	92	84
Succinic acid	100	94	90	87	100	100	96	89
cis-Aconitic acid	100	100	92	88	100	100	100	93
Cysteine (control)[b]	100	100	100	100	100	100	100	100

[a] Medium contains 0.2% Na_2SO_4, 0.002% $MgCl_2$, 0.05% KH_2PO_4, 0.15% K_2HPO_4, and 0.01% cysteine-HCl.
[b] Cysteine furnished the nitrogen for all tubes; note that cysteine cannot furnish both the carbon and nitrogen sources when used alone. 100, no growth; 99–90, poor growth; 89–80, fair growth; 79–70, good growth; and 69–60, excellent growth.

$MgCl_2$, 0.05% KH_2PO_4, 0.2% glucose, and 0.005% *l*-glutamic acid. However, growth was sparse in the latter medium and not suitable for observing an increase in bacterial numbers turbidimetrically. Adding 0.005% concentrations of *l*-aspartic acid and cystine to this medium afforded a suitable test system for this purpose. The relative growth rates of the two strains in nutrient broth and in this latter synthetic medium are given in Figs. 1 and 2, respectively.

It was important to note that, regardless of the nature of the nutritional environment employed in this study, the resistant strain was never stimulated to a level of growth comparable to that of the sensitive strain. This growth lag displayed by the resistant variant is an interesting variational character similar to the slower growth rates described for certain phage-resistant variants (6), and for streptomycin-resistant *Salmonella* (7).

No differences were observed in utilization of glutamic acid and aspartic acid by the two strains, although the resistant strain took twenty to twenty-four hours longer to effect the same degree of utilization as did the sensitive strain. Apparently, the rate of utilization of these two amino acids was commensurate with the relative growth rates of each strain. For a given strain, plots of time *versus* viable counts, and plots of time *versus* amino acid utilization would yield identical curves.

During the amino acid studies, the appearance of a new ninhydrin positive spot on the chromatograms was observed. Subsequent studies proved this new spot to be alanine. Although no attempt was made to quantitate alanine production by the two strains, visual comparisons of spot size, color intensity, and disappearance of glutamate and aspartate were significant in determining whether differences in alanine production existed between the two strains. These observations indicated that the two strains produced alanine at about the same rate, when glutamate and aspartate were used as the substrate.

It was interesting to note that the streptomycin-resistant *S. schottmuelleri* failed to grow in Koser's citrate medium. Growth could not be initiated whether 1×10^4 cells or 1×10^8 washed cells per ml. of medium was used. However, the streptomycin-sensitive *S. schottmuelleri* grew rapidly and profusely in the same medium when only 1×10^4 washed cells per ml. of medium was used, and the strain could be successfully subcultured in the medium.

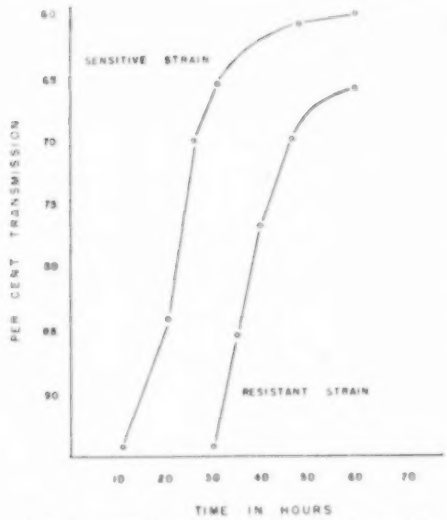

Fig. 2.—Growth curves of streptomycin-sensitive and streptomycin-resistant strains of *S. schottmuelleri* grown in a chemically-defined medium, pH 7.0, at 37°. Medium composed of 0.2% Na_2SO_4, 0.002% $MgCl_2$, 0.05% KH_2PO_4, 0.2% glucose, 0.005% *l*-glutamic acid, 0.005% *l*-aspartic acid, and 0.005% cystine.

In biochemical reactions the resistant strain demonstrated a response forty-eight to seventy-two hours later than the sensitive strain and failed to produce gas from maltose or glucose in seventy-two hours. In contrast, the sensitive strain gave, in twenty-four hours, reactions comparable to those noted in Bergey's Manual (8) for a typical *S. schottmuelleri*. Seligmann and Wassermann (7) reported that their streptomycin-resistant strains of *Salmonella* completely lost the ability to produce gas from carbohydrates, whereas the resistant strain used in this study lost the ability to produce gas only from maltose or glucose among the carbohydrates tested.

SUMMARY

Comparisons of a streptomycin-resistant and a streptomycin-sensitive strain of *S. schottmuelleri* as to differences in relative growth rates, utilization of different carbon and nitrogen sources, and biochemical reactions have been conducted.

The parent strain of *S. schottmuelleri* (ATCC 9282) gave rise to strains which were highly resistant to streptomycin. Resistance to this drug was found to be a relatively stable characteristic. A strain resistant to 5,000 mcg. of streptomycin per ml. of medium has remained resistant to this concentration after four months of repeated subculture in streptomycin-free media.

In general, the two strains displayed qualitative biochemical reactions comparable to those noted in Bergey's Manual (8) for a typical *S. schottmuelleri*. However, in contrast to the parent strain, the resistant strain would not grow in Koser's citrate medium, failed to produce gas from maltose or glucose in seventy-two hours, and consistently exhibited a forty-eight to seventy-two-hour delay in biochemical reactions.

A significant difference in growth was detected between the streptomycin-resistant and streptomycin-sensitive strains. The resistant variant failed to grow in a chemically-defined medium consisting of mineral salts, glucose, and *l*-asparagine which, however, supported growth of the sensitive strain. Regardless of the nature of the nutritional environments employed in this study, the resistant strain was never stimulated to a level of growth comparable to that of the sensitive strain. The resistant variant grew at approximately the same rate in the presence or absence of streptomycin.

No differences were observed in utilization of glutamic acid and aspartic acid by the two strains. Apparently, the rate of utilization of these two amino acids was commensurate with the relative growth rates of each strain. On the basis of qualitative studies, it was believed that alanine was produced at the same rate by both strains

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The Effect of Granule Size Upon Disintegration Time and Capping in Compressed Tablets*

By ALBERT J. FORLANO† and LEONARD CHAVKIN

The effect of compressing several mesh sizes of lactose, sodium bicarbonate, and magnesium trisilicate granulations into tablets was determined. A definite relationship was found to exist between the particle size of the granulation of a particular substance and the disintegration rate, per cent of capping, and other physical tablet characteristics. A definition of the term fines was also proposed.

THE PARTICLE SIZE of the granulated material is an important determinant of the characteristics of the finished compressed tablet. The purpose of this investigation was to determine the effect of compressing various mesh sizes of lactose, sodium bicarbonate, and magnesium trisilicate granulations into tablets, separately. The effect of granule size upon the following characteristics were determined: (*a*) disintegration rate, (*b*) frequency of capping, and (*c*) production problems associated with each mesh size.

The determination of a definition for the commonly used term, fines, and the respective proportion of each mesh size in the granulations were also considered pertinent to this study.

EXPERIMENTAL

Sodium bicarbonate, lactose, and magnesium trisilicate were chosen as the materials in this study because they represent varying degrees of solubility, and will be referred to as the "active ingredients."

A preliminary study was conducted by granulating the three active ingredients with 10% acacia, 50% glucose, and syrup-alcohol (equal parts), in order to determine the most suitable binders. Ten per cent acacia in water was found to be suitable for lactose and sodium bicarbonate and 50% syrup U. S. P.-alcohol was chosen for magnesium trisilicate.

Procedure.—The three active ingredients and corn starch were dried in an oven at 120° F. for three days prior to use. After drying, fifteen pounds of each active ingredient was mixed with 2.65 pounds of corn starch (the active ingredient represented 85% of the mixture and the corn starch 15%). This mixture was passed twice through a Fitzpatrick comminuting machine fitted with a 120-mesh screen. The resultant material passed through a 200-mesh screen.

The powder mixture was placed in a Stokes mixer model 21-H, mixed for one hour, and subsequently granulated with the respective binders. The wet material was passed through a Stokes oscillating granulator model 43-A, fitted with a No. 6-mesh screen, and dried at 120° F. for one week. The dried material was rescreened.

The dried granulation was fractionated by the use of a mechanical shaker and a set of U. S. Bureau of Standard sieves. The following fractions were collected: a control consisting of the original granulation, 6–8, 8–10, 10–12, 12–16, 16–20, 20–30, 30–40, 40–60, 60–80, 80–100, 100–140, 140–200, and 200 mesh and finer (Table I). The fractions were lubricated with 1% of calcium stearate.

TABLE I.—PERCENTAGE OF EACH MESH FRACTION BASED ON THE TOTAL WEIGHT OF GRANULATION[a]

Mesh Size	Magnesium Trisilicate	Sodium Bicarbonate	Lactose
6–8	2.6	..	0.5
8–10	1.6	8.8	5.5
10–12	5.3	8.1	6.7
12–16	17.1	16.6	14.8
16–20	20.8	13.9	13.6
20–30	16.3	12.6	11.7
30–40	19.3	11.1	9.9
40–60	8.6	10.3	13.0
60–80	2.6	4.4	7.3
80–100	1.1	1.9	4.3
100–140	1.0	2.4	4.9
140–200	0.7	1.6	2.5
200 and finer	3.0	8.4	5.9

[a] Five hundred grams of the original granulations were removed before fractionation and used as a control.

Each granulation fraction was compressed on a Stokes model B-2 rotary tablet machine. The pressure was controlled by the use of the safety mechanism, which is essentially a spring attached to the lower pressure wheel. This spring was set to release upon receiving more than four tons of pressure, therefore if that pressure was exceeded, any pressure in excess of four tons would be lost due to the release of the spring. The tablets were compressed with the spring constantly releasing itself. The compression time for each fraction was set at forty to fifty seconds to minimize the possibility of spring fatigue.

One set of $^{13}/_{32}$ inch standard concave punches and dies was used for the compression. After the machine was set up it was allowed to operate, and the pressure was slowly increased until the noise indicating that the safety spring was releasing pressure in excess of four tons was just perceptible. The tablets were compressed under these conditions.

RESULTS

Disintegration Rates.—Six tablets were chosen represented by a weight of 0.50 ± 0.02 Gm. and were subjected to U. S. P. disintegration tests (1).

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The disintegration tests on magnesium trisilicate tablets showed that (with the exception of the 200-mesh and finer fraction) they would not disintegrate completely and left a small, flat, hard core. Therefore, the end point for these tablets was the flat, hard core stage. A plot of disintegration time *versus* mesh size are found in Figs. 1 and 2.

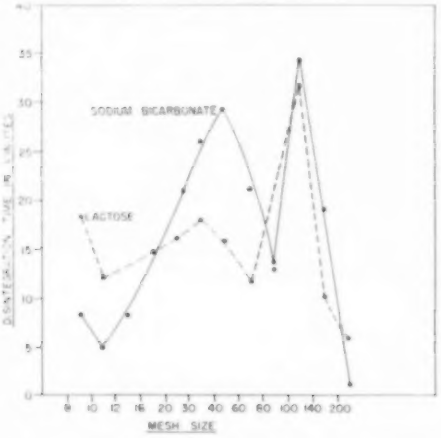

Fig. 1.—Disintegration time for tablets of sodium bicarbonate and lactose of various mesh sizes.

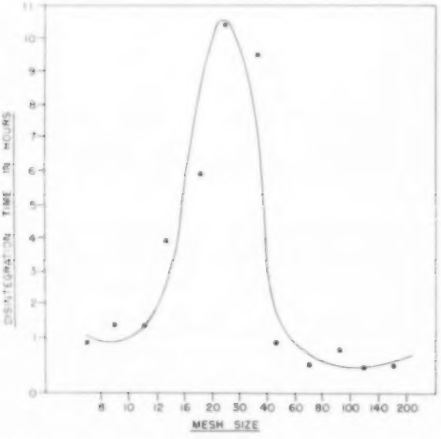

Fig. 2.—Disintegration time for tablets of magnesium trisilicate of various mesh sizes.

The average U. S. P. disintegration rates of two runs were used in plotting the data. These values are given in Table II.

Per Cent of Capping.—This was determined by placing a jar containing the tablets into a mechanical shaker and allowing them to shake for five minutes. Then the contents of the jars were weighed and the amount of capped material was determined by weighing the amount that would pass through a No. 10 screen. The per cent of capping was determined by Eq. 1. This information is presented in Fig. 3.

TABLE II.—U. S. P. DISINTEGRATION RATES FOR SODIUM BICARBONATE, LACTOSE, AND MAGNESIUM TRISILICATE TABLETS

Mesh Size	Sodium Bicarbonate, min.	Lactose, min.	Magnesium Trisilicate, hr.
8–10	8.5	19.5	0.81
10–12	5.0	12.5	1.40
12–16	8.2	13.0	3.80
16–20	14.5	14.5	6.00
20–30	21.0	16.0	10.50
30–40	26.0	18.0	9.58
40–60	29.0	16.0	0.91
60–80	21.0	11.5	0.61
80–100	14.0	13.5	0.71
100–140	34.0	31.5	0.53
140–200	19.0	10.2	0.40
200 mesh and fines	1[a]	6.0	0.32

[a] These tablets crumbled upon ejection.

$$\frac{\text{Weight of capped material}}{\text{Weight of entire sample}} \times 100 = \text{Per cent capping} \quad \text{(Eq. 1)}$$

Per Cent of Each Size in Total Granulation.—The distribution of the different mesh sizes in the granulations were recorded and tabulated in Table I. These results are not to be construed as showing a general pattern because they can be affected by many variables present in the granulation of a tablet mass.

DISCUSSION

It was found, generally, that tablets made from granules larger than 16 mesh, possessed a veined structure. This was probably due to the lack of fine particles to fill the intergranular spaces. The particles of 200 mesh and finer of soluble materials

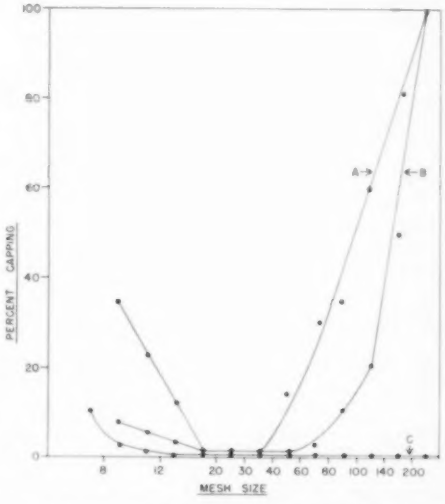

Fig. 3.—Per cent capping found among tablets of various mesh sizes of lactose, sodium bicarbonate, and magnesium trisilicate (respectively, *A*, *B*, and *C*).

did not form tablets upon compression, but resulted in capped masses.

Sodium Bicarbonate.—The incidence of capping was least in the range of 16–40 mesh; however, capping increased with decreasing mesh size following 60 mesh. This increased capping might be explained by the fact that granules, finer than 60 mesh, were not as well granulated as the larger granules, and under compression their granule structure was crushed resulting in a loss of cohesion. No tablets could be made using 200-mesh and finer granules.

The disintegration data showed that tablets made, using granules of 16–60 mesh, had the longest disintegration times.

As the granule size decreased below 60 mesh the disintegration time decreased with an unexpected rise at 100–140 mesh, followed by a decrease at 140–200 mesh (Fig. 3). An increase in capping was also exhibited in the 60 to 200 mesh and finer range. Tablets made from granules smaller than 60 mesh lost the glossy appearance that was characteristic of the larger mesh sizes.

Lac ose.—The behavior of the lactose granulations closely followed the pattern exhibited by sodium bicarbonate granulations. However, there was a minor difference; disintegration time and per cent of capping indicated the lactose granules were becoming weaker at levels smaller than 40 mesh. The comparable change with sodium bicarbonate occurred at 60 mesh.

Magnesium Trisilicate.—The tablets made using 12 to 30-mesh granules had longer disintegration times than the smaller mesh sizes. When tablets were made from granules smaller than 30 mesh the disintegration time decreased, and per cent of capping was negligible.

Examination of the disintegration rates shows that the disintegration time decreased from ten hours beginning with 30–40 mesh, to nineteen minutes at 200 mesh and finer. Only the tablets made from 200 mesh and finer granules were found to disintegrate completely; in all other cases the tablets left a small, hard core. The core might be explained by Johnston (2) who stated that the center of a compressed body consists of a matted, hard core. This core formation, accentuated by the insoluble nature of the material, probably prevented the water from penetrating into the center of the tablet to effect complete disintegration.

The decrease in disintegration time observed as the smaller particles were compressed was opposite to the trends observed with the other granulations of this study.

It is possible that the nature of the bonding occurring either in the granules or in forming the lactose and sodium bicarbonate tablets was different from that in the magnesium trisilicate tablets. It is also conceivable that the effects observed may have been due to the different actions of acacia and sucrose as binders. However, from the data collected in this investigation it was not possible to offer an adequate explanation for the anomalous behavior of the magnesium trisilicate granulations.

Fines.—As a result of this study it was possible to formulate a definition of fines for the particular substances tested here. Sodium bicarbonate and lactose showed that the disintegration time decreased, binding of punches and dies increased, and per cent of capping increased in the vicinity of 40 to 80 mesh. Since an excess of these particles and smaller ones were detrimental to the compression of satisfactory tablets they may be considered fines.

In the compression of the magnesium trisilicate tablets, particles smaller than 60–80 mesh caused binding between the punches and the dies and were therefore considered fines.

SUMMARY

1. The relationship between the particle size of the granulated material and disintegration time and capping of resultant tablets was demonstrated.

2. Sodium Bicarbonate.—Tablets compressed using granules in the range of 8 to 40 mesh exhibited a rise in disintegration time and capping decreased as 60 mesh was approached. With tablets compressed using granules of 60 mesh and smaller, the disintegration time decreased and capping increased; this trend continued through 200 mesh and finer, at which point tablets could no longer be made. The optimum granule size for compression of sodium bicarbonate tablets appeared to be 16 to 60 mesh.

3. Lactose.—These tablets exhibited the same characteristics as sodium bicarbonate tablets except that the transition to poorer tablets occurred after 40 mesh. Consequently, the optimum particle size for lactose granulations was found to be in the 16–40 mesh range.

4. Magnesium Trisilicate.—The per cent capping decreased as the mesh size went from 16 to 200 mesh and finer. At 200 mesh and finer the per cent of capping was smallest. From 30–200 mesh and finer the disintegration time decreased Due to binding of dies and punches with sizes below 60 mesh, the best mesh sizes for these tablets were 16 to 60 mesh.

5. Fines.—Granules of 40–60 mesh and smaller were considered fines in the sodium bicarbonate and lactose granulations: granules of 60 mesh or smaller can be considered fines in the magnesium trisilicate granulations.

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Pressurized Pharmaceutical Aerosols for Inhalation Therapy I*

Physical Testing Methods

By IRVING PORUSH, CHARLES G. THIEL, and JAMES G. YOUNG

The physical testing methods used for controlling the manufacture of pressurized aerosols for inhalation therapy are described. Results of the tests for uniformity of valve delivery, particle size, and pressure of this new pharmaceutical dosage form are reported. A reliable and reproducible aerosolized dose of medication can be administered for inhalation therapy by means of the metering valve on the pressurized container.

THE INTRODUCTION of pressurized aerosols for inhalation therapy provided a new pharmaceutical dosage form for dispensing a measured quantity of medication in the precise particle size required for optimum results. Clinical evaluation of the effectiveness and acceptability by users of this form of medication are reported in the literature (1–6). A number of advantages of packaging in pressurized containers are described by Sciarra (7), Root (8), and Barr (9). Among the more compelling ones are protection of contents from atmospheric oxidation, moisture, and bacterial contamination; and accurate delivery of a measured dose of aerosolized drug. The reliability of dispensing accurate dosage by this means, as with other pharmaceuticals, is largely dependent upon the controls and specifications imposed on its manufacture. Physical measurements of particle size, pressure, and uniformity of valve delivery are new criteria for efficacy that must be rigorously controlled to achieve a dependable pharmaceutical preparation. A description of the methods and techniques employed in the physical testing of Medihaler[1] pressurized aerosols is presented here.

METHODS AND TECHNIQUES

Particle Size Analysis.—The efficacy of inhalation aerosols is as dependent upon particle size as on the concentration of medicament. There is general agreement in the literature that optimum particle diameter for penetration into the pulmonary depths is between one-half and seven μ (10–12). Smaller particles are exhaled and larger ones do not penetrate to the desired depth. Determination of particle dimensions must be accurate, reproducible, and sufficiently rapid for routine application.

Methods employed in the analysis of particles from pressurized containers are dependent, to some extent, on whether they are solutions or suspensions in propellent. The solution of drugs in fluorohydrocarbons usually requires the use of a cosolvent (e. g., alcohol), in about 50% of the volume. Sprays of these solutions take several seconds for the higher boiling cosolvent to evaporate, and the particles change dimensions as vaporization occurs. Any measurement of these particles in this dynamic system must take into account the rate of evaporation, and correlate this rate with the time necessary for inspiration of medication.

The Cascade impactor described by Pilcher, *et al.* (13), and Tarpley (14) is most suitable for measurement of the particle size of solution-type aerosols. This method depends on the principle that in an airborne stream of particles projected through a series of nozzles and glass slides at increasing velocity, larger particles are impacted first on the lower velocity stages, while smaller particles pass on and are collected on later stages. The most serious deficiency of this apparatus is the relatively large sample needed for analysis. This makes determinations of particles emanating from single doses of most aerosols impossible by ordinary analytical methods. Measurements of particle size were made with the Cascade impactor and the distribution determined on an isoproterenol aerosol solution containing 0.25% w/w isoproterenol HCl in a propellent with 35% w/w (50% v/v) alcohol. The mass median diameter was 7 μ; 45% of the mass was less than 5, 50% less than 7, and 58% less than 10 μ.

Preparations, such as the Medihaler, containing suspensions of micronized drug in liquefied propellent without a cosolvent, present a different problem in particle size analysis. The boiling point of the propellent mixture is about −17°, resulting in almost instantaneous vaporization on reaching atmospheric pressure, and leaving the solid particles suspended in the air-gas stream. Measurement of these particles can be made more readily by use of almost any sizing apparatus, and more important, can be checked by microscopic observation. A rapid, reproducible apparatus for measurement of small particle size distribution is described by Dimmick, *et al.* (15). The technique used is termed "the light scatter decay method," and involves a simplified analysis of the change in light intensity of a Tyndall beam as an aerosol settles under turbulent conditions. Only one or two measured doses of the aerosol are required, making possible its application to the study of stability of particle dimensions on prolonged storage.

The distribution of particles is usually reported by giving the mass median diameter as found in the light

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[1] Medihaler-Iso (Riker) is a suspension of 2 mg./cc. isoproterenol sulfate in an inert propellent. Medihaler-Epi (Riker) is a suspension of 7 mg./cc. epinephrine bitartrate in an inert propellent.

scatter decay apparatus. In addition, it is of interest to determine the per cent of the mass of the material in particles less than 5 μ, less than 7 μ, and less than 10 μ. The specification established in this laboratory for material acceptable for use in inhalation therapy is "the mass median diameter determined on the light scatter decay apparatus is 4 μ or less, and 85% or more of the mass is less than 7μ in diameter." Typical analyses of recent runs of Medihaler-Iso and Medihaler-Epi aerosols are shown in Table I.

TABLE I.—PARTICLE SIZE OF ISOPROTERENOL AND EPINEPHRINE AEROSOL SUSPENSIONS

Lot No.	Mass Median Diameter, μ	Mass of Particles with Diameters <5 μ, %	<7 μ, %	<10 μ, %
ISO-91782	3.5	70	88	98
ISO-91795	2.7	78	92	99
ISO-91794	2.9	77	92	99
EPI-91797	3.5	73	93	100

An important consideration in the manufacture of pressurized pharmaceutical aerosol suspensions is the stability of particle dimensions on prolonged storage. To demonstrate that growth of the microscopic particles does not occur in Medihaler-Iso and Medihaler-Epi, their particle size was determined at various intervals after manufacture on the light scatter decay apparatus. Results are shown in Table II.

TABLE II.—STABILITY OF PARTICLE DIMENSIONS OF MEDIHALER-ISO AND MEDIHALER-EPI

Lot No.	Storage Period, 22°	Mass Median Diameter, μ	Mass of Particles with Diameters <5 μ, %	<7μ, %	<10 μ, %
ISO-X9012	Initial	2.5	79	94	98
ISO-X9012	3 months	2.7	82	95	99
ISO-X9012	6 months	2.5	83	96	99
ISO-71240	18 months	3.0	77	92	99
EPI-71288	18 months	3.2	75	92	99

Valve Delivery.—Uniformity of dose from the pressurized aerosol is dependent upon the reproducibility of the valve delivery. Inasmuch as this is a mechanical device, and the quantity delivered is only 50 μL. per depression, very fine tolerances must be set in production of the valve components to achieve good reproducibility.

Dosage is controlled by taking a statistical sample of every finished lot of valves and testing for delivery. The weight before and after a single valve depression is recorded and converted to volume by calculation from the known density of the suspension. Variability of dose from an individual valve and from valve to valve is very small. Measurements of recent lots of Medihaler-Iso and Medihaler-Epi aerosols are shown in Tables III and IV.

Specifications for acceptance of Medihaler vials include tolerances for valve delivery as determined on the finished product. All valves must deliver between 85 and 115% of the calculated 50 μL. dosage.

Pressure.—Particle size is related in varying degrees to the internal pressure of both solution- and suspension-type aerosols. For solutions, the wetness of spray and particle size are almost completely dependent upon internal pressure. Temperature and pressure changes have a profound effect on the distribution of particles emitted by solution-type aerosols with alcoholic cosolvents. Rapid expansion and vaporization of propellant leaves the relatively high boiling alcohol to form droplets which must evaporate to yield the small particles. Mechanical devices are used to aid the break up of droplets, but pressure is still the predominant factor in determining particle distribution of the solution-type aerosol medication.

Pressure is, to a lesser extent, important in breaking up agglomerates of micronized drug particles of suspension-type aerosols. To illustrate this dependency, determination of particle size distributions for identical micronized powders emitted from containers of 45 psig[2] and 80 psig were made. The data are shown in Table V. Microscopic observation of powder showed some agglomerates, but no individual particles greater than 5 μ.

TABLE III.—VALVE TO VALVE REPRODUCIBILITY OF DELIVERY

Lot	No. Vials Tested	Mean Delivery, μL.	Standard Deviation, μL.	Range, μL.
EPI-91798	50	48.1	±1.6	44.4–53.2
EPI-91797	24	49.8	±1.3	46.5–52.1
ISO-91796	24	51.2	±1.0	47.8–53.6
ISO-91795	24	50.4	±1.4	46.7–52.3
ISO-91794	24	49.4	±1.8	46.1–52.9
ISO-91753	100	51.2	±1.4	45.8–54.3

TABLE IV.—REPRODUCIBILITY OF INDIVIDUAL VALVES

Volume Delivered by Valve at 20-Shot Intervals Until Contents of Vial Are Emptied

	Mean, Delivery, μL.	Standard Deviation, μL.	Range, μL.
Valve 1	48.4	±0.35	48.0–49.0
Valve 2	49.4	±0.32	48.9–49.8
Valve 3	50.1	±0.62	49.3–51.0
Valve 4	49.7	±0.53	49.0–50.5

TABLE V.—EFFECT OF INTERNAL PRESSURE OF ISOPROTERENOL SUSPENSION ON PARTICLE SIZE DISTRIBUTION EMITTED

Sample	Pressure, psig	MMD, μ	Mass with Diameters <5μ, %	<7 μ, %	<10 μ, %
55202	80	1.9	99.6	100.0	100.0
	45	3.7	69.9	89.0	98.6
55200	80	2.5	83.1	95.4	99.6
	45	4.0	66.2	89.0	98.9

Determination of pressure is made by use of an accurate gauge fitted with a hypodermic needle. The container held in a water bath is pierced with the hypodermic needle, the vial and gauge tapped lightly, and reading is made. If a gas tight seal is not made between needle and inside of the container, the aerosol can be cooled to −40°, transferred to a cold 10-cc. plastic-coated glass vial, and sealed with a multiple-dose neoprene stopper and aluminum closure. Pressure readings can then be readily made through the aluminum and rubber closure with the hypodermic needle pressure gauge.

[2] Pounds per square inch, gauge.

CONCLUSIONS

Reproducibility of dosage and consequent uniformity of therapeutic effect of pressurized aerosols for inhalation can be achieved when adequate control is excercised over particle size, valve delivery, and pressure of the finished preparation. Considering that only microgram quantities of medication are delivered, tolerances for variation are comparable to other oral dosage forms. Reproducibility is much superior to oral liquid preparations dispensed by teaspoon or dropper, and compares favorably with weight variation permitted for hard gelatin capsules (16).

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Pressurized Pharmaceutical Aerosols for Inhalation Therapy II*

Analytical Control Methods

By JAMES G. YOUNG, IRVING PORUSH, CHARLES G. THIEL, SHELDON COHEN, and C. HOWARD STIMMEL

Analytical procedures for the control of pressurized aerosols for inhalation therapy require techniques not usually encountered in other dosage forms. Procedures are described for assay of active ingredients, amount of medication delivered per dose, and moisture content. Claims for improved stability of epinephrine and isoproterenol in this new dosage form are supported by data obtained from chemical and biological stability studies. Uniformity of dosage delivered from the aerosol valve and from the adapter was determined by chemical analyses and found to be well within the acceptable limits for other dosage forms.

THE ANALYTICAL CONTROL of pressurized aerosols for oral inhalation therapy involves techniques not usually encountered in other dosage forms. It is the purpose of this communication to describe some of these techniques and to provide data which emphasize two of the advantages of pressurized aerosols, *viz.*, improved stability of medicament and uniformity of dosage.

Data are presented on two aerosols, Medihaler-Epi and Medihaler-Iso, which contain epinephrine bitartrate and isoproterenol sulfate, respectively, suspended in a mixture of fluorochlorohydrocarbons with a surfactant as dispersing agent.[1]

* Received August 21, 1959, from the Riker Laboratories, Inc., Northridge, Calif.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

[1] Medihaler-Epi (Riker Laboratories, Inc.) contains 7 mg. of epinephrine bitartrate per cc. of suspension. Medihaler-Iso (Riker Laboratories, Inc.) contains 2 mg. of isoproterenol sulfate per cc. of suspension.

SPECIFICATIONS OF PROPELLENTS

Identification.—The fluorochlorohydrocarbons used as propellents are identified by infrared spectrophotometric analysis of a completely volatilized sample.

Inorganic Fluoride.—Products used internally must be free of inorganic fluoride, with a maximum limit of 10 p.p.m. considered acceptable. Tests for fluoride ion that could be a product of hydrolysis of the fluorochlorohydrocarbons are essential. The analysis of inorganic fluoride is carried out on an aqueous extract of propellent using the micro method of spontaneous electrolysis according to Baker and Morrison (1).

High Boiling Residue.—A sample of each propellent is evaporated to dryness. The high boiling residue is determined gravimetrically and must not exceed 0.05%.

DETERMINATION OF MOISTURE

One of the factors which contributes to the stability of epinephrine and isoproterenol in this dosage

form is the exclusion of moisture. The maximum tolerance for total moisture is 250 p. p. m. for Medihaler-Iso, which contains 100 p. p. m. as water of hydration, and 150 p. p. m. for Medihaler-Epi.

The method used in this laboratory is that of Karl Fischer (2, 3). A sample of aerosol suspension is added to a measured amount of Karl Fischer (K. F.) reagent and the excess reagent is then back titrated with a standard solution of water in methanol.

Standardization of the K. F. reagent and the titration of the sample are carried out in a clear-glass plastic-coated bottle containing a magnetic stirring bar and sealed with a multiple-dose neoprene closure. To prevent interference from atmospheric moisture during standardization of the K. F. reagent, a positive pressure (*ca.* 5 psig)[2] is maintained in the titration bottle by the addition of a small amount of propellent 114. Addition of the propellent or sample to the titration bottle is accomplished by transfer from a warmed bottle to a chilled bottle using a double-ended 20-gauge hypodermic needle. A mixture of formamide:methanol (1:1, *ca.* 4 ml.) is used to solubilize the propellents in a 4–5-Gm. sample of aerosol suspension.

Titrations are made with hypodermic syringes equipped with 24-gauge needles and graduated to 0.05 ml. or less. The end point of a titration is determined electrometrically using the titrimeter circuit described by Reed (4). The platinum wire electrodes are inserted through the neoprene seal of the titration bottle with the aid of a 20-gauge hypodermic needle.

ASSAY PROCEDURES

Assay of Epinephrine Bitartrate.—The gross weight of the intact aerosol is determined accurately. Using a sharp tool, the top of the closure is punctured and a 25-gauge hypodermic needle is inserted. The gaseous propellent is allowed to escape until the internal pressure has been reduced to a point where the container can be opened safely. The closure is removed and the residual suspension poured onto a small sintered-glass filter of fine porosity. The dispersing agent is removed from the solid epinephrine bitartrate by washing the container and valve with 25 ml. of carbon tetrachloride in several small portions, adding the washings to the filter. After each transfer from container to filter, the solid residue is allowed to settle on the filter with the application of light suction. The filtrate is discarded. The container and valve are washed by the above technique using about 50 ml. of distilled water. The filtrate is collected, transferred quantitatively to a 100-ml. volumetric flask, and brought to volume with distilled water. The absorbance at 278 mμ of an appropriate dilution is compared to that of a standard solution of epinephrine bitartrate U. S. P. The empty aerosol vial and valve are dried and their combined tare weight determined. Using the spectrophotometric data and the net weight of the aerosol, the concentration of epinephrine bitartrate in the aerosol is computed.

The absorption spectrum from 400 to 210 mμ of the final dilution must conform to that of a standard solution of epinephrine bitartrate U. S. P.

The aerosol of isoproterenol sulfate is assayed by the same procedure except that 0.01 *N* sulfuric acid is used as the solvent.

[2] Pounds per square inch, gauge.

STABILITY OF MEDICAMENT

One of the advantages of this aerosol preparation is that the active ingredients are protected from atmospheric oxygen. This is pertinent in the case of the catechol amines which are known to be susceptible to oxidative deterioration. Also, the stability of these compounds is further enhanced by the fact that they are present as completely insoluble suspensions in a nonaqueous medium.

The excellent stability of epinephrine bitartrate and isoproterenol sulfate is demonstrated by the data in Tables I and II. To assure that the spectrophotometric assays are a true measure of active catechol amine, bioassays are included.

TABLE I.—STABILITY OF EPINEPHRINE BITARTRATE

	Per Cent of Initial Concentration		
	12 Months		24 Months
	Room Temperature	45°	Room Temperature
Chemical[a]	100.5	100.4	97.5
Bioassay[b]	98.8	108.3	99

[a] Average of determinations on three aerosols.
[b] Method of U. S. P. XV.

TABLE II.—STABILITY OF ISOPROTERENOL SULFATE

	Per cent of Initial Concentration		
	12 Months		24 Months
	Room Temperature	45°	Room Temperature
Chemical[a]	95.5	91.5	93.5
Bioassay[b]	117.2	118.3	94

[a] Average of determinations on three aerosols.
[b] Depressor effect of isoproterenol was measured using modification of method for epinephrine U. S. P. XIV.

UNIFORMITY OF DOSAGE

In a previous communication (5) data were presented showing the reproducibility of weights of individual shots taken at intervals from a single aerosol. However, since these preparations are suspensions it remained to be proved that a uniform shot-weight represents a uniform dosage of active ingredient. This has been accomplished by chemical assay of the active component delivered in each of several shots taken at regular intervals from an aerosol vial.

Delivery of Active Ingredient from Valve.—The aerosol was placed inverted in the bottom of a 250-ml. glass-stoppered cylindrical graduate containing exactly 200 ml. of 0.1 *N* acetic acid. Using a glass rod pressed against the bottom of the vial, the aerosol was discharged by pressing the stem of the valve against the bottom of the graduate. The rod was carefully dropped into the graduate which was quickly stoppered and inverted twenty-five times. This technique has the advantage of extracting the gas phase which appears as a cloud above the surface of the liquid when the aerosol is discharged. A 10-ml. aliquot of the acetic acid solution was assayed for catechol amine by the method of Weil-Malherbe and Bone (6) using the fluorimetric attachment of the Beckman DU spectrophotometer equipped with

a primary filter, Wratten No. 50, and a secondary filter, Wratten No. 15(G). With each aerosol, every tenth shot was taken arbitrarily with a total of 21–22 shots being collected and assayed. This procedure effectively empties the aerosol. The results are shown in Table III.

TABLE III.—UNIFORMITY OF DOSAGE

Mcg. Epinephrine Bitartrate/Valve Depression	Mcg. Isoproterenol Sulfate/Valve Depression
312	104
307	104
339	97
393	97
344	101
295	99
335	98
329	96
342	97
339	91
326	96
306	93
296	97
301	95
323	94
344	97
312	97
325	93
293	98
285	100
394	95
360	
Mean 327.3	Mean 97.1
S. D. 29.3	S. D. 3.3

Delivery of Active Ingredient from Adapter.—Oral inhalation of a drug from an aerosol requires the use of a special adapter which is inserted into the mouth before discharging the aerosol. A certain amount of drug is trapped by the adapter, thereby reducing the amount of drug delivered to the patient. To determine the amount of drug trapped by the adapter and the uniformity of dose delivered to the patient, the apparatus pictured in Fig. 1 was used. The aerosol with adapter was discharged into a funnel connected by Tygon tubing to a glass tube with a sintered plate immersed in a gas-washing bottle containing exactly 75 ml. distilled water. A second tube from the gas-washing bottle was connected to a vacuum line which pulled 16 L./min. of air through the system as the aerosol was discharged. Thus, each dose was delivered through the adapter under conditions emulating inhalation by the patient. After each shot, the solution in the gas-washing bottle was transferred to a 200-ml. volumetric flask. The funnel, tubing, and bottle were washed with several portions of distilled water and the washings added to the volumetric flask. Sufficient glacial acetic acid was added to the flask to give a 0.1 *N* solution when brought to volume. A 10.0-ml. aliquot of the final solution was assayed fluorimetrically as described previously. Every tenth shot with a total of ten shots was collected and assayed. The results are shown in Table IV.

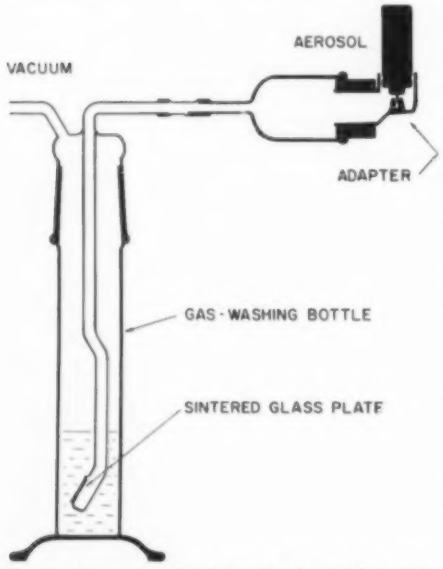

Fig. 1.—Apparatus for determination of dose delivered from adapter.

TABLE IV.—UNIFORMITY OF DOSAGE THROUGH ADAPTER

Epinephrine Bitartrate Delivered From Adapter per Valve Depression, mcg.	
250	302
280	265
246	331
250	268
243	287
Mean 272	
S. D. 28	

The average amount of drug delivered through the adapter was 77.9% of the theoretical amount calculated from volume delivered by the valve.

SUMMARY

Pressurized aerosols for the administration of drugs have received considerable acclaim because of their many advantages. However, the literature has given little attention to the means whereby these advantages become manifest, particularly in aerosols which are made to deliver small doses of potent drugs for internal use. The latter require the most exacting standards for their control and manufacture.

The data presented here and in a previous communication (5) represent an attempt to establish some standards for this dosage form.

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A Comparative Study of Pharmaceutical Emulsification Equipment*

By GARNET E. PECK, H. GEORGE DEKAY, and GILBERT S. BANKER

A study has been made of six common types of emulsification equipment normally encountered in pilot plant experimentation or small-scale manufacturing. Mineral oil and cod liver oil emulsions were prepared. The evaluation of the emulsion products involved measurements of particle size distribution, rate of creaming, and viscosity. The particle size distribution studies and the rate of creaming evaluation clearly differentiated the equipment studied. It was shown that the emulsions processed with a homogenizer usually produced samples with a smaller average particle size and a lower rate of creaming under the conditions of the study. The data of this study were subjected to statistical evaluation which indicated that both the emulsifying agent and the processing equipment used significantly affect emulsion quality. A significant interaction between equipment and emulsifying agents was also shown.

THE PREPARATION and evaluation of liquid emulsified products present unique and important problems to the pharmaceutical industry. To economically manufacture stable and pharmaceutically elegant emulsion products, the best power equipment should be selected for the required processing, and then this equipment should be used in an efficient manner.

In this investigation six common types of emulsification equipment were used to prepare various emulsion samples. The samples were observed for their (*a*) degree of creaming, (*b*) apparent viscosity, and (*c*) particle size distribution of the dispersed phase. The results were then statistically correlated to the equipment utilized for the various formulations studied.

EXPERIMENTAL

Emulsification Equipment and Standardization.—The equipment used in this study may be divided into the three groups as stated by Johnson (1). Table I illustrates the equipment used and the conditions under which they were operated. The wall mixer was equipped with a 34.5-cm. shaft, a 7.6-cm. two blade marine-type propeller, and a 1/4-hp motor, which was mounted vertically on a movable frame. The container used with this mixer had a diameter of 20.3 cm. and a height of 24.5 cm. The mixer shaft was vertically inserted 5.5 cm. from the edge of the container. For the Eppenbach Homo Mixer, which had a 5.5-cm. propeller, a container with a diameter of 16.3 cm. and a height of 19.3 cm. was used. The mixing shaft was inserted vertically and placed at the center of the container during the mixing.

In this study the r. p. m. of all equipment except the Manton Gaulin homogenizer were recorded and maintained at a definite level. The measurements were taken with a Strobatac.[1] For the standardization of both equipment and emulsion preparation it was necessary to prepare preliminary emulsions.

Table I.—Equipment Speeds and Settings

Equipment	Speed, r. p. m.	Setting
Agitators		
Wall mixer[a]	1,700[b]	...
Waring Blendor[c]	10,000	...[d]
Eppenbach Homo Mixer[e]	1,700 and 7,000[b]	...
Colloid Mills		
Tri-Homo disperser[f,g]	7,000	0.005 and 0.003 in.
Eppenbach colloid mill[f,e]	7,000	0.005 and 0.003 in.
Homogenizer		
Manton Gaulin homogenizer[f,h]	...	3,000 and 8,000 p. s. i.

[a] Eastern Engineering Co., New Haven, Conn.
[b] Mixing time, five minutes for final volume.
[c] Waring Products Corp., New York 36, N. Y.
[d] Mixing time, three minutes.
[e] Gifford-Wood Co., New York 17, N. Y.
[f] Emulsions processed through equipment once.
[g] Tri-Homo Corp., Salem, Mass.
[h] Manton-Gaulin Manufacturing Co., Inc., Everett, Mass.

From these emulsions the equipment speeds and settings were established for this study as shown in Table I. The settings in Table I for the Manton Gaulin homogenizer were for the first valve. For the second stage valve the setting used was 500 p. s. i. in all cases. For all the processing equipment the samples were collected while the equipment was at top speed or at a constant pressure.

Particle Size Evaluation.—A direct microscopic measurement of the globule diameter of the dispersed phase was performed using a stand microscope equipped with a 15× eyepiece and a 43× objective. The eyepiece contained a micrometer with a 5-mm. horizontal scale divided into 100 divisions. In order to examine the emulsions microscopically it was necessary to dilute them with some liquid which would not affect the dispersed phase, but would decrease Brownian movement. A 50% aqueous solution of propylene glycol was used for this purpose, based on the findings of Levius and Drommond (2). The samples were diluted in a standard spot plate from ten to twentyfold, the finer emulsions requiring the larger dilution. One drop of the diluted emulsion was then placed on a microscope slide, a cover glass was placed over the drop, and the slide allowed to stand at least fifteen minutes on the microscope stage before viewing.

* Received August 21, 1959, from Purdue University, School of Pharmacy, Lafayette, Ind.
Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.
[1] General Radio Corp., Cambridge, Mass.

To determine the sample size (number of particles to be measured) in order to obtain a reasonably accurate estimation of population parameters, the method of Harris, Horvitz, and Moon (3) was applied to preliminary data representing the range of emulsion products anticipated for the study. With the test a maximum confidence interval of one limit, a 99% confidence coefficient, and a 95% probability were employed, with 99 degrees of freedom. A sample size of 400 particles or globules was indicated as adequate under these test conditions, and 400 particle size measurements consequently were made to obtain each emulsion mean particle size.

Viscosity Measurements.—In order to observe any change in apparent viscosity between samples or after aging, a Brookfield Synchroelectric, Model RVF viscometer[2] was used with the No. 1 spindle at a speed of 10 r. p. m. As indicated by Tober and Autian, the viscometer was operated for ten minutes in the sample before a reading was taken (4). All measurements were made at 25°.

Formulation.—The first emulsion prepared was the official liquid petrolatum emulsion N. F. X (5). However, it was necessary to reduce the quantity of acacia from the official 12.5% to 5.0% as suggested by Levius and Drommond (2). The emulsions prepared with power equipment were much too thick when the higher percentage of acacia was used. For the second official emulsion (cod liver oil emulsion N. F. X) the acacia was also reduced from 12.5 to 5.0% (6). The third emulsion prepared was an emulsion of liquid petrolatum using a mixture of 1.5% Tween 80[3] and 3.5% Arlacel 80[3] as the emulsifying agent. The fourth emulsion was a liquid petrolatum emulsion using the U. S. P. method for emulsification (7).

Manufacturing Directions for Emulsions.—With the exception of the U. S. P. gelatin method the emulsions were mixed as follows: (*a*) The oil and the emulsifying agent were mixed for one minute at 1,000 r. p. m. (*b*) The proper volume of water was then added all at once. The primary emulsions were formed at 1,700 r. p. m. over a three-minute period (7,000 r. p. m. used for the Eppenbach Homo Mixer). (*c*) The remaining ingredients were added, water added to volume desired, and then mixed for five minutes at 1,700 r. p. m. (7,000 r. p. m. used for the Eppenbach Homo Mixer).

For the liquid petrolatum emulsions prepared by the gelatin method the prescribed U. S. P. procedure was followed. The final mixing of these emulsions was accomplished at 1,700 r. p. m. for the wall mixer and 7,000 r. p. m. for the Eppenbach Homo Mixer.

Machine Processing Schedule.—Table I gives the setting and speeds used for the various samples. All samples that were processed with the refining equipment were passed through the equipment once, as this is the ideal method of equipment utilization, permits more absolute equipment comparison, and establishes a standard procedure which may be easily maintained.

Tube Sedimentation Method.—A variety of methods for the study of sedimentation or creaming of emulsified products was found in the literature. In this study Pyrex 20-mm. external diameter tubing was cut into 18-inch lengths. To close the tubes, rubber stoppers, size No. 3 were used. Each tube was calibrated to 100 ml. using a 100-ml. volumetric flask and distilled water. These tubes were stored at room temperature in special tube racks.

Photomicrographs.—To supplement the particle size measurements photomicrographs were taken using a standard microscope and an Eastman Kodak attachment.[4] The magnification of the microscope was 430×. The exposure times most frequently employed were three and five seconds.

RESULTS AND DISCUSSION

In order to present the results of this study more efficiently, it is necessary to use a coding system for the oils, emulsifying agents, and equipment utilized in this work. Table II explains the various symbols used.

Temperature of Emulsions After Processing.—During the preparation of the emulsions the processing equipment was standardized for use as described under *Experimental*. As a portion of the equipment evaluation, the temperature rise produced during processing was considered. The temperature of each emulsion was taken immediately following its preparation. Two pieces of equipment were equipped with cooling jackets: the Tri-Homo disperser and the Eppenbach colloid mill. The room temperature was 25°. Table III illustrates the temperatures of the emulsions after processing.

From Table III it can be seen that the Eppenbach Homo Mixer at 7,000 r. p. m. produced the greatest temperature rise in all of the emulsions studied. No apparent changes due to the temperature differences were observed.

Apparent Viscosity Studies.—All emulsion viscosity measurements were performed twenty-four hours after emulsion preparation or processing. The viscosity measurements were repeated after one

TABLE II.—CODING OF SAMPLES AND EQUIPMENT

Material	Coding	Definition
Oil	LP	Liquid petrolatum
Oil	CD	Cod liver oil
Emulsifier	A	Acacia
Emulsifier	TA	Tween 80-Arlacel 80
Emulsifier	G	Gelatin
Equipment	WM	Wall mixer
Equipment	WB	Waring Blendor
Equipment	T5	Tri-Homo disperser, 0.005 in.
Equipment	T3	Tri-Homo disperser, 0.003 in.
Equipment	E5	Eppenbach colloid mill, 0.005 in.
Equipment	E3	Eppenbach colloid mill, 0.003 in.
Equipment	MG3	Manton Gaulin homogenizer, 3,000 p. s. i.
Equipment	MG8	Manton Gaulin homogenizer, 8,000 p. s. i.
Equipment	HM 1,700	Eppenbach Homo Mixer, 1,700 r. p. m.
Equipment	HM 7,000	Eppenbach Homo Mixer, 7,000 r. p. m.

[2] Brookfield Engineering Laboratories, Stoughton, Mass.
[3] Atlas Powder Co., Wilmington 99, Del.
[4] Eastman Kodak Co., Rochester, N. Y.

TABLE III.—EMULSION TEMPERATURES AFTER PROCESSING, ° C.

Emulsions	Equipment									
	WM	WB	T5	T3	E5	E3	MG3	MG8	HM 1,700	HM 7,000
LP-TA	28	28	25	25	25	25	32	39	29.5	42
LP-A	27	30	25	25	25	25	29	38	29.8	41
LP-G	34	32	24	24	23	23	31	37	...	38
CD-A	28	30	25	25	25	25	31	40	...	48

month of aging at room temperature. Viscosity measurements of the emulsions prepared with acacia or the Tween-Arlacel combination were also made after two months of aging. In general, there were no appreciable changes in apparent viscosity after aging. The emulsions from the LP-G series showed little variation in apparent viscosity as a result of the equipment used. When emulsions of liquid petrolatum and cod liver oil were prepared with acacia, the Eppenbach Homo Mixer produced the least viscous product.

Rate of Creaming Studies.—To obtain a rate of creaming for the emulsion samples, the samples were observed during shelf aging in the tubes at room temperature and the degree of separation recorded by the measurement of the aqueous layer. This measurement was then plotted against time as illustrated by Fig. 1. From graphs such as this, the sedimentation or creaming velocity was calculated according to Stokes' law. The results of the sedimentation velocity study are given in Table IV. These values have been evaluated with caution since the slope of some of the curves was determined from a line with only two points. It can be said that the Manton Gaulin homogenizer at 8,000 p. s. i. produced emulsions which had the slowest rate of creaming in all cases and thus produced the best appearing emulsions on aging. In the case of the cod liver oil emulsions the degree of creaming was greatly reduced in all samples. It was not possible to determine the sedimentation velocity for the liquid-gelatin series due to a delayed separation of the emulsion samples.

Particle Size Measurements and Their Statistical Evaluation.—Following the measurement of 400 globules of each emulsion studied, the average particle size and the standard deviation of the samples were determined. Tables V and VI are the summation of all these determinations. It can be seen that in most cases those samples prepared initially with the wall mixer had the largest average apparent particle size. Differences in the average particle size between the readings were shown not to be statistically significant.

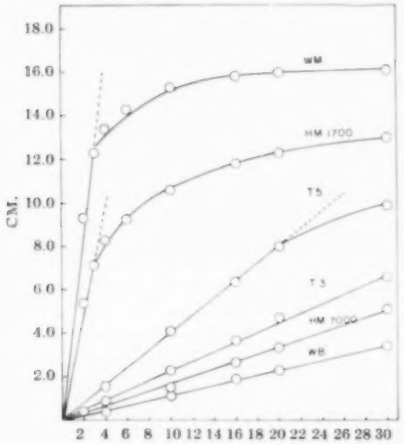

Fig. 1.—Rate of creaming, LP-A series.

TABLE IV.—SEDIMENTATION VELOCITIES OF THE EMULSIONS IN CM./DAY

Equipment	LP-A Series V	LP-TA Series V	CD-A Series V
WM	4.10	5.00	1.50
WB	0.12	0.80	0.01
T5	0.40	2.10	0.30
T3	0.23	1.96	0.14
E5	0.24	2.10	0.30
E3	0.21	1.97	0.11
MG3	0.17	0.76	0.01
MG8	0.12	0.00	0.01
HM 1,700	2.37	1.18	...
HM 7,000	0.17	2.90	0.33

Example, LP − A − WM: $V = \frac{h_2 - h_1}{t_2 - t_1} = \frac{12.3 - 0}{3.0 - 0} = 4.10$

An analysis of variance was performed to assist in the interpretation of the particle size distribution studies. A three-factor analysis of variance was conducted, with i = 1–3 for the three emulsifying agents, j = 1–2 for the two aging periods (initially and one month later), and k = 1–8 for the equipment and settings utilized, according to the i, j, k notation and design of Ostle (8). Experimental replication indicated good reproducibility of the results. It should be noted that the results from the wall mixer and the Eppenbach Homo Mixer at 1,700 r. p. m. were not included. These samples had rather large average particle sizes and thus were obviously significantly different from all other samples. Table VII illustrates the results of the analysis of variance. From the table it can be seen that at the 5% level, significant differences are indicated between emulsifying agents, between equipment, and with the equipment-emulsifying agent interaction. In other words, the analysis of variance indicates that there are significant differences in average emulsion particle size depending on which emulsifying agent is used, which piece of processing equipment is used, and which piece of processing equipment is used with which emulsifier. The differences in average particle size with aging were not statistically significant, nor were the reading interactions significant.

To determine individual differences in average emulsion particle size of various pieces of processing equipment with each formulation studied, the Newman-Kuels test was employed (9). This test is valuable for making multiple nonindependent signifi-

TABLE V.—PARTICLE SIZE MEASUREMENTS FOR THE LP-A AND LP-TA SERIES

Sample	1st Reading[a]		2nd Reading[b]		3rd Reading[c]	
	$\bar{X}$[d]	S[e]	$\bar{X}$	S	$\bar{X}$	S
LP-A-WM$_f$	13.452	15.026	9.516	18.847	16.643	20.533
LP-A-WB	5.294	2.560	5.312	1.369	5.447	1.672
LP-A-T5	5.191	7.341	5.745	3.138	4.214	2.631
LP-A-T3	4.018	2.271	3.941	2.391	3.734	2.156
LP-A-E5	5.857	7.080	7.507	6.854	5.921	3.898
LP-A-E3	5.100	1.897	6.361	7.277	5.426	2.802
LP-A-MG3	3.562	1.990	2.801	1.940	4.058	4.131
LP-A-MG8	4.453	1.761	4.875	3.498	4.904	5.059
LP-A-HM 1,700	13.485	12.515	7.036	7.438	4.342	7.197
LP-A-HM 7,000	3.815	1.390	3.799	3.063	3.367	0.976
LP-TA-WM	4.190	6.816	2.223	2.601	1.056	1.984
LP-TA-WB	2.600	5.366	2.133	0.616	1.700	0.672
LP-TA-T5	5.070	1.819	2.785	2.458	1.843	2.454
LP-TA-T3	4.410	3.003	4.103	3.354	1.593	2.118
LP-TA-E5	3.900	2.698	2.855	3.724	2.033	1.678
LP-TA-E3	4.300	3.719	1.748	2.032	1.892	1.885
LP-TA-MG3	1.400	0.399	1.320	0.478	1.086	0.324
LP-TA-MG8	1.030	0.262	0.930	0.442	0.918	0.175
LP-TA-HM 1,700	3.880	3.139	2.228	2.600	1.520	3.233
LP-TA-HM 7,000	2.091	2.223	1.815	0.488	1.457	0.531

[a] Initial measurement. [b] After one month. [c] After two months. [d] In microns. [e] Standard deviation. [f] See Table II for code.

TABLE VI.—PARTICLE SIZE MEASUREMENTS FOR THE LP-G AND THE CD-A SERIES

Sample	1st Reading[a]		2nd Reading[b]	
	$\bar{X}$[c]	S[d]	$\bar{X}$	S
LP-G-WM[e]	19.148	18.763	19.288	17.622
LP-G-WB	2.823	3.173	3.782	3.503
LP-G-T5	5.881	6.582	6.820	11.554
LP-G-T3	7.090	12.314	6.585	11.133
LP-G-E5	4.245	6.654	6.149	7.998
LP-G-E3	6.058	7.265	3.542	3.771
LP-G-MG3	1.384	1.004	1.303	0.965
LP-G-MG8	1.426	0.289	1.122	0.274
LP-G-HM 7,000	3.326	2.843	3.413	2.502
CD-A-WM	5.864	7.597	8.596	8.441
CD-A-WB	1.769	0.748	1.568	0.846
CD-A-T5	4.489	2.508	5.301	4.169
CD-A-T3	3.970	1.399	4.015	2.762
CD-A-E5	2.823	1.590	3.469	1.658
CD-A-E3	2.868	2.064	4.210	1.631
CD-A-MG3	1.841	0.322	1.915	0.401
CD-A-MG8	2.296	0.028	2.474	0.288
CD-A-HM 7,000	2.159	0.989	2.075	0.801

[a] Initial measurement. [b] After one month. [c] In microns. [d] Standard deviation. [e] See Table II for code.

cance tests on differences among individual means in an analysis of variance. In the case of the liquid petrolatum-acacia emulsions after the first reading the following was shown: (*a*) No significant difference between the Waring Blendor and the Tri-Homo disperser at 0.005-inch samples was observed. (*b*) No significant difference between the Tri-Homo disperser at 0.005-inch and the Eppenbach colloid mill at 0.003 inch- samples was observed.

For the remainder of the samples in the series, all particle size differences between the equipment utilized were significant.

Photomicrographs.—Photomicrographs are presented to illustrate the particle distribution of the emulsions. In all the samples the emulsions were diluted with the same quantity of the 50% aqueous propylene glycol solution. Figure 2 is the photograph of the wall mixer sample of a liquid petrolatum-Tween-Arlacel emulsion. Figure 3 is the photograph of this formulation processed with the Manton Gaulin homogenizer at 8,000 p. s. i. From these two figures it can be seen that there is a definite increase in the number of globules per unit area as the size of the globules is reduced.

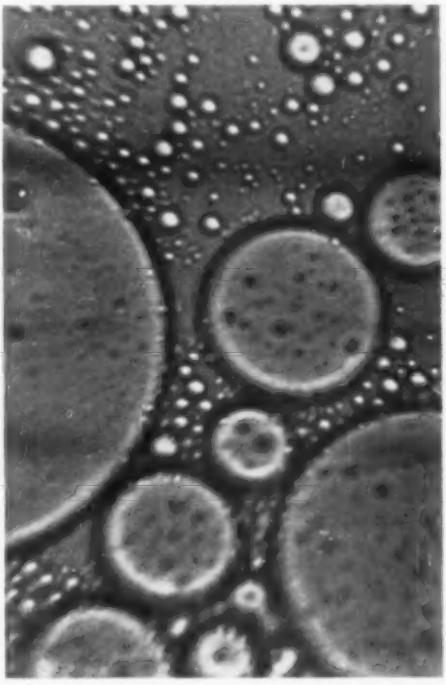

Fig. 2.—Liquid petrolatum—Tween, Arlacel emulsion: wall mixer.

TABLE VII.—ANALYSIS OF VARIANCE

Source	Sum of Squares	Degrees of Freedom	Mean Square	Calculated F	$F_{0.05}$[a]	Significant
i (Agents)[b]	39.5764	2	19.7882	34.7222	3.74	Yes
j (Readings)[c]	0.2670	1	0.2670	0.4685	4.60	No
k (Equipment)	71.7397	7	10.2485	17.9830	2.77	Yes
$i \times j$	3.4820	2	1.7410	3.0549	3.74	No
$i \times k$	28.8507	14	2.0608	3.6160	2.48	Yes
$j \times k$	3.6306	7	0.5187	0.9102	2.77	No
$i \times j \times k$ (error)	7.9788	14	0.5699	...	...	...
Total		47	...	...	...	...

[a] $F_{0.05}$ = 5% Points from the F distribution (9). [b] Emulsifying agents. [c] The effect of aging.

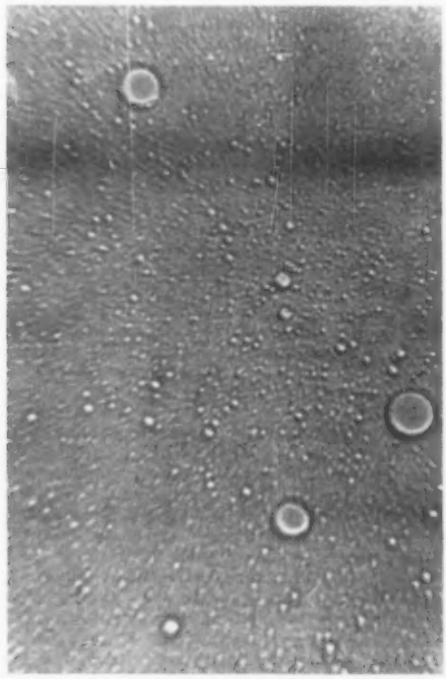

Fig. 3.—Liquid petrolatum—Tween, Arlacel emulsion: Manton Gaulin homogenizer, 8,000 p. s. i.

CONCLUSIONS

1. When utilizing certain pieces of equipment to process several emulsion formulations, an appreciable emulsion temperature increase occurred. The Eppenbach Homo Mixer produced the greatest temperature increase, 20°.

2. Emulsions prepared with acacia were more viscous than those prepared with either gelatin or a Tween-Arlacel combination. Some differences in apparent viscosity were observed when the various pieces of equipment were utilized. Aging of the emulsion samples at room temperature did not produce appreciable changes in apparent viscosity.

3. When using a simple sedimentation or creaming test conducted at room temperature, it was shown that the Manton Gaulin homogenizer at 8,000 p. s. i. produced the least amount of creaming in the emulsions. The rate of creaming was calculated according to Stokes' law.

4. The Manton Gaulin homogenizer was the most effective of all the equipment studied, using a decrease in particle size of the dispersed phase as the criterion for efficient emulsification processing. The homogenizer produced samples with the smallest average particle size for all four formulations studied.

5. An analysis of variance of the mean particle size data indicated that significant differences existed between (*a*) the three emulsifying agents, (*b*) the six pieces of processing equipment, and (*c*) the interaction between the emulsifying agents and equipment.

6. With the formulations used in this study, one pass through the homogenizer (pressure type) produced a much greater particle size reduction than any other piece of equipment used.

7. A refined oil such as cod liver oil emulsified with acacia is definitely easier to emulsify than liquid petrolatum with the same emulsifying agent, as shown by the smaller average particle size of the wall mixer samples and by the general lower rate of creaming.

In the preparation of emulsions, the emulsifying agents and the equipment to be utilized should be carefully considered in any formulation studies to obtain maximum particle size reduction.

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The Synthesis and Pharmacology of N-(Substituted Aminoacyl)-chlorotoluidines II*

By ELIAS EPSTEIN and NICHOLAS MALATESTINIC

A number of N-(substituted aminoacyl)-chlorotoluidines were prepared and tested for local anesthetic potency, toxicity, and irritation on laboratory animals. Several of these compounds, as well as a few previously described, were tried clinically for local anesthesia in dental procedures.

THE PROPERTY of inducing local anesthesia is widely spread among the various nitrogen-containing organic compounds. A great number of amino derivatives of esters, amides, alcohols, and hydrocarbons have been prepared and their local anesthetic effect noted in the literature.

In our continuing search for better local anesthetics, we had described in our previous paper (1) several N-(substituted aminoacyl)-chloroanilines and toluidines, some of which showed sufficient promise to warrant further investigation of this chemical structure. Additional compounds of this type, as well as a few N-ethyl chlorotoluidines, were prepared. After having been tested for anesthetic potency, toxicity, and for irritation on laboratory animals, several compounds from this as well as from the previously prepared group were tried clinically for local anesthesia in dental procedures.

The general method of preparation consisted of treating a chloroacyl chloride with a chlorotoluidine in a manner described by Jacobs and Heidelberger (2), with some modifications. The chlorotoluidide was treated with excess amine to form the anesthetic base which was isolated and purified by distillation under high vacuum of between 10 and 40 μ. The anesthetics were isolated as their hydrochloride salts. Table I lists the boiling point of the bases and the melting point and analysis of the chlorotoluidide hydrochlorides.

EXPERIMENTAL

N-Ethyl-3-chloro-4-methylaniline. — A mixture of 169 Gm. (1.2 mole) of 3-chloro-4-methylaniline, 166 Gm. of diethyl sulfate, and 960 ml. of 2 *N* sodium hydroxide was heated at reflux temperature, with stirring, for one hour. After the mixture was saturated with sodium chloride, the upper oil layer was separated and dissolved in 500 ml. of 3 *N* hydrochloric acid. A solution of 83 Gm. of sodium nitrite in 500 ml. of water was added slowly, with stirring. The reaction temperature was kept under 10°. The precipitate which formed was filtered and washed consecutively with water, 3 *N* hydrochloric acid, and then water. The dried precipitate was dissolved in 200 ml. of ethyl alcohol. The solution was saturated with hydrogen chloride and heated at reflux temperature for two hours. On evaporation of the alcohol, 75 Gm. (37%) of crude N-ethyl-3-chloro-4-methylaniline hydrochloride was obtained as a white solid.

α - Chloro - 3 - chloro - 4 - methyl - N - ethylacetanilide.—To 75 Gm. (0.3 mole) of crude N-ethyl-3-chloro-4-methylaniline hydrochloride in 250 ml. of glacial acetic acid was added, with stirring, 40 Gm. (0.36 mole) of chloroacetyl chloride. The mixture was stirred for ten minutes and 200 Gm. of sodium acetate, dissolved in 500 ml. of water, was added all at once, with strong stirring. The slurry, after stirring for thirty minutes, was filtered and washed with water. The precipitate was dissolved in ether and the solution was extracted with dilute ammonium hydroxide and then water. After having been dried over anhydrous sodium sulfate, the ether solution was distilled. The fraction at 125–135° at 70 μ yielded 60 Gm. (72%) of the anilide as a light yellow oil.

Illustrative General Procedure for Table I Compounds.—A mixture of 15 Gm. (0.06 mole) of α-chloro-3-chloro-4-methyl-N-ethyl-acetanilide and 22 Gm. (0.3 mole) of isobutylamine was heated at reflux temperature for twelve hours. The excess isobutylamine was evaporated on a steam bath and the residue treated with excess 5% hydrochloric acid. The mixture was extracted three times with ether. The water layer was made alkaline with excess ammonium hydroxide and extracted with ether. The ether solution was distilled and the fraction boiling at 110–120° at 20 μ yielded 9.8 Gm. (56%) of a clear liquid. An ether solution of the base was neutralized with anhydrous hydrochloric acid and the precipitate recrystallized from ethyl acetate to yield α-isobutylamino-3-chloro-4-methyl-N-ethyl-acetanilide hydrochloride as white crystals, m. p. 164–165°.

PHARMACOLOGY

A pharmacological screening for use as local anesthetics of these compounds as well as those previously published (1) was conducted and several compounds were selected for clinical testing.

The topical anesthetic potency was determined by application to the rabbit cornea of successively lower concentrations of each compound and comparing the length of anesthesia with that given in the literature for cocaine hydrochloride (3). This method, originated by Koller and described by Hirschfelder and Bieter (4) was modified as described in a previous paper (5).

The conductive anesthetic potency was determined by blocking the sciatic nerve of the guinea pig

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The authors are indebted to Richard Sriubas for assistance in the analyses and to Michael Fisher for assistance in the pharmacological testing of these compounds.

TABLE I.—N-(SUBSTITUTED AMINOACYL)-CHLOROTOLUIDINES $RNHCO(CH_2)_nR'$

R′	B. p., °C./μ Base	n	M. p., °C. HCl	Formula	Ionic Cl, % Calcd.	Ionic Cl, % Found	Molecular Wt. Calcd.	Molecular Wt. Found
			(1) R = 2-Methyl-3-chlorophenyl					
$NHCH_3$	120/20	1	Decompn. 233	$C_{10}H_{14}ON_2Cl_2$	14.24	14.30	249	246
NHC_3H_7	140/20	1	Decompn. 273	$C_{12}H_{18}ON_2Cl_2$	12.78	12.95	277	278
NHC_2H_5	161/40	2	204–206	$C_{12}H_{18}ON_2Cl_2$	12.78	12.58	277	281
NHC_4H_9	149/30	1	Decompn. 235	$C_{13}H_{20}ON_2Cl_2$	12.18	12.32	291	289
$NH(CH_2)_3OCH_3$	...	1	216–218	$C_{13}H_{20}O_2N_2Cl_2$	11.51	11.78	307	285
Piperidino	143/30	1	227–229	$C_{14}H_{20}ON_2Cl_2$	11.69	11.86	303	288
NHC_4H_9(iso)	168/20	2	229–230	$C_{14}H_{22}ON_2Cl_2$	11.62	11.68	305	309
$N(C_2H_5)_2$	155/20	2	135–137	$C_{14}H_{22}ON_2Cl_2$	11.62	11.48	305	296
$NH(CH_2)_3OCH_3$	205/20	2	123–126	$C_{14}H_{22}O_2N_2Cl_2$	11.04	10.97	321	316
N′ Methyl piperazino	...	1	Decompn. 235	$C_{14}H_{22}ON_2Cl_2$	19.99[a]	20.38	355[a]	349
$N(C_3H_7)_2$	130/10	1	162–164	$C_{15}H_{24}ON_2Cl_2$	11.10	11.08	319	319
$N(C_4H_9)_2$(iso)	145/20	1	165–166	$C_{17}H_{28}ON_2Cl_2$	10.19	10.34	348	347
			(2) R = 4-Methyl-3-chlorophenyl					
NHC_2H_5		1	246–248	$C_{11}H_{16}ON_2Cl_2$	13.50	13.26	263	266
NHC_4H_9		1	263–265	$C_{13}H_{20}ON_2Cl_2$	12.18	11.86	291	290
NHC_4H_9(*tert*)		1	Decompn. 281	$C_{13}H_{20}ON_2Cl_2$	12.18	12.28	291	291
NHC_4H_9		2	216–217	$C_{14}H_{22}ON_2Cl_2$	11.62	11.61	305	305
$N(CH_3)C_4H_9$		2	175–176	$C_{15}H_{24}ON_2Cl_2$	11.10	11.23	319	316
			(3) $C_2H_5NCOCH_2R'$					
NHC_2H_5	105/40		177–178	$C_{13}H_{20}ON_2Cl_2$	12.18	12.42	291	291
NHC_4H_9(iso)	110/20		164–165	$C_{15}H_{24}ON_2Cl_2$	11.10	11.12	319	319
$N(C_2H_5)_2$	102/10		145–146	$C_{15}H_{24}ON_2Cl_2$	11.10	11.20	319	319

[a] Dihydrochloride.

TABLE II.—PHARMACOLOGY OF N-(SUBSTITUTED AMINOACYL)-ANILIDES

R	R′	n	Relative Potency: Topical[a]	Relative Potency: Conductive[b]	Relative Potency: Infiltration[b]	Rel. Toxicity[c]: i. p.	Rel. Toxicity[c]: s. c.	Irritation[d]: Eye	Irritation[d]: Wheal	Clinical Evaluation[e]
3-Chloro	NHC_2H_5	1	C	B	B	0.7	0.8	0	0	C
3-Chloro	$N(C_2H_5)_2$	1	C	B	A	0.7	0.5	0	0	B
3-Chloro	$N(C_2H_5)_2$	2	C	A	B	0.6	0.8	0	0	..
3-Chloro	$NH(CH_2)_3OCH_3$	1	C	B	B	0.8	1.1	0	0	B
2-Methyl-3-chloro	NHC_2H_5	1	C	B	A	0.5	0.4	+	0	B
2-Methyl-3-chloro	$N(C_2H_5)_2$	1	C	B	B	0.8	0.5	0	0	C
2-Methyl-3-chloro	$N(C_2H_5)_2$	2	A	C	A	1.7	2.0	0	0	.
2-Methyl-3-chloro	NHC_4H_9(iso)	1	C	B	A	0.5	0.6	0	0	..
2-Methyl-3-chloro	NHC_4H_9(iso)	2	B	C	A	1.3	2.0	+	+	..
2-Methyl-3-chloro	Piperidino	1	B	B	B	1.0	0.9	0	0	..
2-Methyl-4-chloro	$N(C_2H_5)_2$	2	C	B	B	0.4	0.5	0	0	C
2-Methyl-5-chloro	$NH(CH_2)_3OCH_3$	1	B	A	B	1.7	1.3	0	0	B
2-Methyl-6-chloro	NHC_4H_9(*tert*)	1	A	A	A	1.7	2.0	0	0	..
4-Methyl-3-chloro	NHC_3H_7	1	B	B	A	0.5	0.5	0	0	B
4-Methyl-3-chloro	NHC_3H_7	2	B	A	A	0.6	0.5	0	0	B
4-Methyl-3-chloro	NHC_4H_9(iso)	1	B	A	A	0.3	0.3	+	0	..

[a] As compared with cocaine hydrochloride, A—more potent, B—approx. equal, C—less potent.
[b] As compared with procaine hydrochloride, A—more potent, B—approx. equal, C—less potent.
[c] As compared with procaine hydrochloride, the value for the LD_{50} s. c., 750 mg./Kg.; i. p., 250 mg./Kg.
[d] Little or no irritation, 0; moderate irritation, +; severe irritation, ++.
[e] As compared with procaine hydrochloride, A—more potent, B—approx. equal, C—less potent.

with successively lower concentrations of each compound and comparing the depth and length of anesthesia to that obtained with procaine hydrochloride. Although this method originally introduced by Shackell (6) approximates conditions found in clinical practice, it has been found in some instances to give an erroneous value for the anesthetic potency in humans (5).

The infiltration anesthetic potency was obtained by noting the anesthetic effect of an intradermal injection on the guinea-pig back under controlled conditions (7) and comparing the lowest concentration that will produce anesthesia under these conditions as compared with procaine hydrochloride.

The subcutaneous and intraperitoneal toxicities were determined on white mice and the upper limits of the intravenous toxicities were obtained using rabbits. Each compound was checked for irritation, both on application to the rabbit eye and on intradermal injection in the rabbit skin, by the trypan-blue test of Weatherby (8).

The concentrations of the compounds prepared for the clinic varied from 0.5 to 2% with epinephrine 1:50,000. The upper limit used was determined by the toxicity. No clinical solution more toxic than 2% procaine hydrochloride was used.

The pharmacological results of sixteen of the more promising compounds out of a total of seventy-two, together with a preliminary clinical evaluation of nine of these are listed in Table II. The results should be considered as semiquantitative since, because of the large number of compounds screened, a minimum number of animals per compound was used. An average of four, six, and eight tests were used to obtain the relative topical, conductive, and infiltration potency of each compound. An average of twenty mice per compound were used to determine the subcutaneous and intraperitoneal toxicity. The preliminary clinical evaluation was based on an average of twenty injections in the various concentrations for each compound, with both block and infiltration type injections being used, in a variety of dental procedures.

DISCUSSION AND SUMMARY

Twenty additional N-(substituted aminoacyl)-chlorotoluidines were synthesized and they, together with the fifty previously prepared compounds of this type, were tested pharmacologically for use as local anesthetics. Of the nine tested clinically, six proved to be approximately equal in potency to procaine hydrochloride in dental procedures. Several compounds were precluded from clinical testing because of their low solubility in isotonic solution.

Although it was difficult to correlate molecular structure with physiological activity, it was noted that, in general, those compounds more potent topically were also more toxic and that the substitution of the ethyl group for hydrogen on the aromatic nitrogen atom resulted in more irritating and less efficient local anesthetics.

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Preparation of Compressed Tablet Granulations by the Air-Suspension Technique II*

By DALE E. WURSTER†

A new and rapid method for preparing compressed tablet granulations by the air-suspension technique is discussed. Experimental data dealing with material losses and variations in the drug and water content of the prepared granulations are presented.

MUCH INVESTIGATIONAL EFFORT has been expended in attempts to find means for preparing compressed tablet granulations which are devoid of the inherent disadvantages of the conventional methods. The wet method, although probably the most widely employed, has the main disadvantage of exposing the drugs to water or other solvents and usually requires a long drying time. Also, it is composed of several individual operations including blending, granulating, and grinding which necessitates a large amount of material handling. The precompression method usually eliminates the need for wetting the drugs but blending and grinding procedures and a considerable amount of material handling are still present. In addition, the method is not suitable for many drugs which are not readily compressible.

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U. S. Patent applied for.
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The air-suspension granulating method discussed in this paper is an attempt to circumvent some of the disadvantages of the above methods. It is extremely rapid and eliminates the need for separate drying, dry blending, and grinding procedures. As currently used, this method is essentially a particle build-up rather than a particle aggregation process. However, if particle aggregation is desired, it too can be obtained by increasing the humidity in the column.

Previous exploratory work (1, 2) showed that it was possible to apply common granulating materials to small particles and then immediately place the formed granules in a tablet machine without further treatment. Therefore, this paper deals mainly with such aspects as the loss of material during operation and the drug and water concentration in the finished granules rather than a detailed description of the process.

EXPERIMENTAL

The procedure employed in preparing the granulations was similar to that previously described (1) for the air-suspension coating technique. Solid particles were coated with common granulating materials while suspended in an upwardly moving air stream in a vertical column. The apparatus used in this process is shown in Fig. 1.

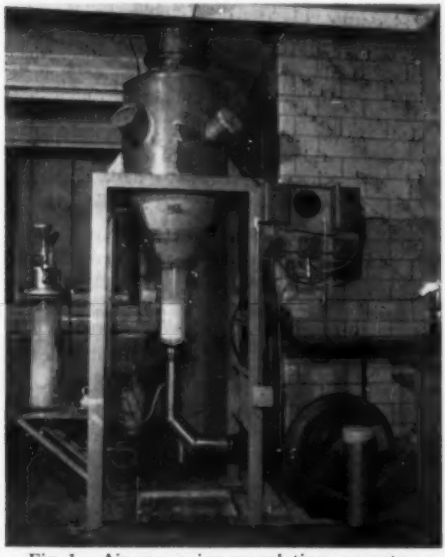

Fig. 1.—Air-suspension granulating apparatus.

In this work 3 to 4 Kg. of 30- or 40-mesh particles were introduced into the column and coated with 2 to 2.2 Kg. of granulating solids. In this case sugar particles served as the nuclei of the resulting granules; however, the nucleus can be composed of either inert material or a drug. When the nucleus is a drug the granulating materials can be applied to the surface. Conversely, with an inert nucleus the drug can be included in the coat. With the above procedures nearly any drug concentration can be obtained in the granulation.

A variety of granulating materials from both aqueous and organic solvent systems have been applied to solid particles. The coating fluid used in this work was composed of 1,000 Gm. sodium bicarbonate, 496 Gm. lactose, 471 Gm. starch, 8 Gm. sodium lauryl sulfate, 500 ml. of 5% starch paste, and 500 ml. water. The sodium bicarbonate served as a test substance for assay purposes.

Since this investigation was concerned only with the aforementioned quantitative aspects of this process none of the granulations prepared for this series of tests were subjected to the tabletting process. Also, no attempt was made to find formulas which would give better bonding to the nucleus or yield satisfactory tablets.

Operating Conditions.—In the last four tests shown in Table I an attempt was made to predetermine operating conditions to achieve a 90% humidity in the column. A psychrometric chart was used for this purpose. For example, with wet and dry bulb temperatures of 71° F. and 80° F., respectively, for the air entering the blower, a 140° F. temperature for the inlet air to the column, an air flow of 60 c. f. m. in the column, an 88.5° F. exhaust temperature, and a 90% humidity condition in the column, an approximate drying capacity of 0.0508 lb. of water per minute was obtained. Thus, 3 L. of a coating fluid containing 2.2 lb. of water was atomized in 43.3 minutes (69.3 ml./min.). For a condition of 80% humidity in the column with an exhaust temperature of 91.5° F., other conditions remaining the same, an approximate drying capacity of 0.0487 lb. of water per minute and an atomization rate of 66.3 ml. per minute were obtained.

Methods of Analysis.—The loss of solids was determined from the weight difference between the total solids introduced and the solids recovered from the column. This loss was assumed to be due to the entrainment of fine particles in the exhaust.

The per cent deviation of the sodium bicarbonate content of the finished granulation from the calculated amount was determined with the U. S. P. XV assay method.

The moisture content was determined from the difference between the initial weight of the samples of the granulation and the weight obtained after drying in a vacuum oven at 55° for fourteen hours.

The granulations were classified according to mesh size using standard seives and a Ro-Tap testing sieve shaker.

RESULTS AND DISCUSSION

Loss of Solids.—In Table I data on the loss of solids, the drug content, and the moisture content are presented. The last four tests were performed with predetermined conditions (90% humidity), as previously described, rather than by a trial and error method. From these data it appeared that the loss of solids increased as the humidity in the column and the moisture content in the finished product decreased. The formation of the fine particles which were lost due to entrainment in the exhaust was prob-

TABLE I.—DATA ON MATERIAL LOSS AND DRUG AND WATER CONCENTRATIONS IN A SERIES OF COMPRESSED TABLET GRANULATIONS

Test No.	Mesh Size of Starting Solids	Weight of Total Solids Introduced into Column, Gm.	Weight of Solids Recovered from Column, Gm.	Solids Lost, %	Gm. of $NaHCO_3$ per Gm. of Product		Deviation of $NaHCO_3$ Content, %	Moisture in Granulation, %
					Calcd.	Found		
1	30	6,000	5,863	2.76	0.1667	0.1632	− 2.09	1.02
2	40	6,000	5,550	7.50	0.1667	0.1378	−17.33	0.54
3	40	6,000	5,845	2.58	0.1667	0.1557	− 6.05	1.18
4	20–30	5,000	4,834	3.32	0.2000	0.1988	− 0.60	1.97
5	10	6,000	5,956	0.88	0.1667	0.1652	− 0.89	3.13
6	30	6,200	6,010	3.06	0.1613	0.1601	− 0.74	1.87
7	40	6,200	5,772	6.09	0.1613	0.1574	− 1.42	1.15
8	30	6,200	6,100	1.66	0.1613	0.1639	+ 1.61	1.98
9	30	6,200	6,094	1.71	0.1613	0.1558	− 3.41	2.49
10	40	6,200	6,089	1.79	0.1613	0.1687	+ 4.58	3.33
11	30	6,200	6,111	1.48	0.1613	0.1593	− 1.23	1.20

ably due to a combination of attritional and spray drying effects at low column humidities. Although these losses can be recovered from the exhaust this was not done in this study. When the exhaust temperature approached that calculated for the dew point, aggregation of particles was observed. The formation of fines is also indicated in Fig. 2 where the particle size distribution of the starting material (approx. 40 mesh) and the two extremes, based on the water content (test 2, 0.54% H_2O and test 10, 3.33% H_2O) are shown. In test 10, evidence of particle growth is shown whereas in test 2, no appreciable particle growth occurred and the fines increased. It is also pointed out that a considerable amount of material was lost (Table I) which would increase the per cent of fines in test 2.

Drug Content.—Assays on the sodium bicarbonate content of the finished granulation tend to indicate that with optimum operating conditions satisfactory drug concentrations can be obtained by this method. Unfortunately, the moisture content for the starting materials was not determined. This may possibly account for the large deviation in the sodium bicarbonate content (+4.58%) observed in test 10 since these values were corrected for the water content in the finished product. Other large deviations, especially tests 2 and 3, correlate fairly well with the low moisture content of the granulation.

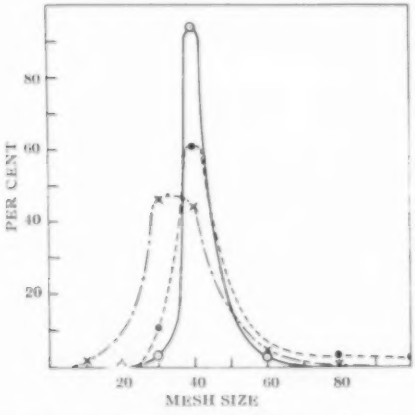

Fig. 2.—A plot of the size distribution of uncoated and coated particles. ⊙———uncoated particles; ● - - - - coated particles, test no. 2, 0.54% water; × — · —coated particles, test no. 10, 3.33% water.

Water Content.—Experimental evidence indicates that the water content of the granulation can be controlled within the limits which are satisfactory for the subsequent tabletting process. In this series of tests, using vacuum oven drying, the water content varied between 0.54 and 3.33% with varying column conditions. In another series of six tests the water content, as determined with a Cenco moisture balance, varied between 0.9 and 2.2%.

As the ratio of the weight of applied solids to the weight of the nucleus increased, improved flow properties of the solid were noted. For example, when the applied solids on sugar crystals (30 mesh, angle of repose 36°) in the form of cubes constituted 10% of the final weight, the original crystalline form tended to be maintained. However, when the applied solids constituted 50% of the final weight, the particles tended to become somewhat spherical in shape (angle of repose 28°) and the flow properties appeared to be enhanced.

A subsequent paper will deal more specifically with the fundamentals of the process and methods of predicting operating conditions for different solvent systems.

SUMMARY

A new method for preparing compressed tablet granulations by a process similar to the air-suspension coating technique is discussed. Based on the experimental evidence obtained it appears that the described process can be controlled to maintain material losses during the operation of the column and the drug and water content of the finished granulation within reasonable limits.

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Physical Properties of Lipids Used in Pharmacy I*

Screening Raw Materials Via Photomicrography

By DAVIS R. REESE, CLIFFORD W. CHONG, and JOSEPH V. SWINTOSKY

A simple photomicrographic technique has been devised to screen lipids for integrity of their crystal structure. In this technique, thin films of the materials are prepared on microscope slides and photomicrographs are taken of their surface and internal structures. The samples are then stored at normal or exaggerated temperatures for any desired period of time. At various intervals, the exact areas appearing in the original photomicrographs are rephotographed and compared with the originals. These permanent records permit detection of subtle changes which occur during aging. The technique has been used with a number of individual materials and simple mixtures. Representative photomicrographs are presented showing the structural integrity of carnauba wax, cetyl alcohol, hydrogenated soya oil, lauryl monoethanolamide, stearyl alcohol, and white wax.

THIS PAPER describes a photomicrographic procedure for discerning the physical integrity of lipid substances. The procedure is illustrated with data from thin films of carnauba wax, cetyl alcohol, hydrogenated soya oil, lauryl monoethanolamide, stearyl alcohol, and white wax.

Many pharmaceutical dosage forms contain one or more fats or waxes. A number of problems associated with these dosage forms are directly related to changes that occur in their lipid components upon aging. For example, changes may occur which affect the hardness of suppositories, the spreadability of ointments, and the absorption of drug from the dosage form.

A program has been established in our laboratory to study lipid or lipid-containing materials. Emphasis in the program is on the nature and degree of physical change these materials undergo after storage. We have found a photomicrographic procedure especially useful to screen the materials for their physical integrity. This screening procedure is designed to detect lipids which are likely to undergo sufficient physical change to alter the elegance and efficacy of dosage forms derived from them. The basic assumption of the test is that the nature and degree of physical change in thin lipid films can be used in predicting the physical changes these same lipids may undergo in dosage forms.

In our procedure, photomicrographs are taken before and after storing the thin lipid films at different temperatures. Comparisons of identical fields on the photomicrographs reveal crystal transformations, crystal growths, or the development of microscopic cracks.

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The authors wish to acknowledge the assistance of Mr. Peter N. Nordberg in preparing the samples.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

EXPERIMENTAL

Apparatus.—The basic items needed for micro observations are a polarizing microscope with a graduated mechanical stage, an adequate light source, a camera with a suitable holder, and a photometer. Additional requirements for macro observations are a low magnification system and a suitable light source.

The equipment utilized in our laboratory consisted of a Leitz Panphot camera microscope[1] equipped with polarizing filters and a graduated mechanical stage, a polaroid camera back containing type 44 film,[2] a built-in filament lamp for the micro observations or a ring illuminator for the macro observations, and a Photovolt photometer model 501-M.[3] Three lens systems were used to study the materials included in this communication: Micro system No. 1, periplanatic ocular 10× + apochromatic objective 24/0.65; Micro system No. 2, Huygens ocular 10× + achromatic objective 10/0.25; and Macro system No. 1, Summar lens f 4.5/8 cm. Actual magnification on the photograph for the three systems was 500×, 200×, and 8×, respectively.

Raw Materials.—Carnauba wax, yellow Grade 1, E. A. Bromund Co.; cetyl alcohol, N. F., M. Michel and Co., Inc.; hydrogenated soya oil (Textee Flakes), E. F. Drew and Co., Inc.; lauryl monoethanolamide (Kessco X-159), Kessler Chemical Co.; stearyl alcohol, U. S. P., E. I. du Pont de Nemours and Co.; and white wax, U. S. P., Koster-Keunen, Inc.

Preparation of Slides for Microscopic Observations.—Thin lipid films, about one crystal thick, make the best samples for microscopic examination.

Crystal changes develop more readily if the films remain uncovered. The films are prepared in the form of wedges to assure the presence of areas approaching optimal thickness. At the time of observation, each wedge is scanned to locate the most desirable field.

Preparation of the slides according to the following procedure has been found satisfactory. First, place a small piece of the test material on a glass slide and

[1] E. Leitz, Inc., New York 16, N. Y.
[2] Polaroid Corp., Cambridge 39, Mass.
[3] Photovolt Corp., New York 16, N. Y.

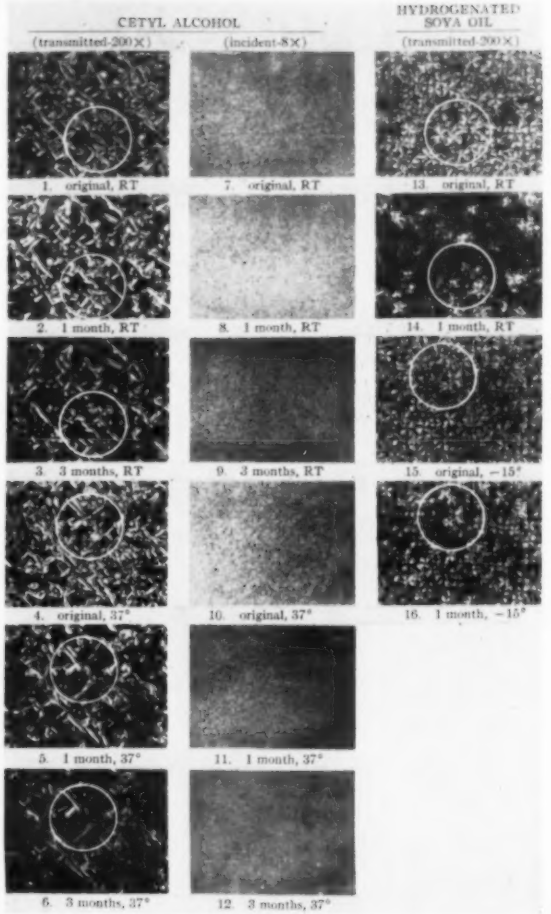
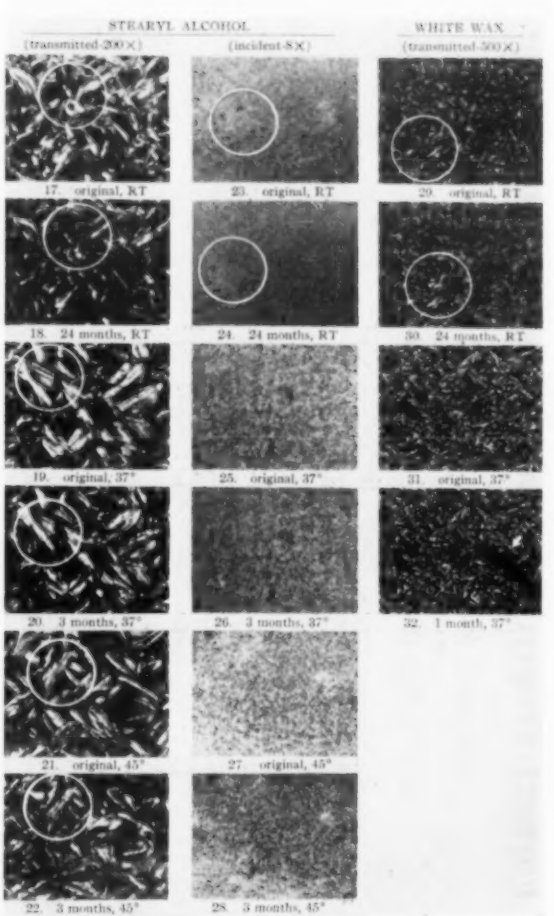


Fig. 1 (*left*).—Photomicrographs showing the crystal structure of cetyl alcohol and hydrogenated soya oil. (RT denotes room temperature.)

Fig. 2 (*right*).—Photomicrographs showing the crystal structure of stearyl alcohol and white wax.



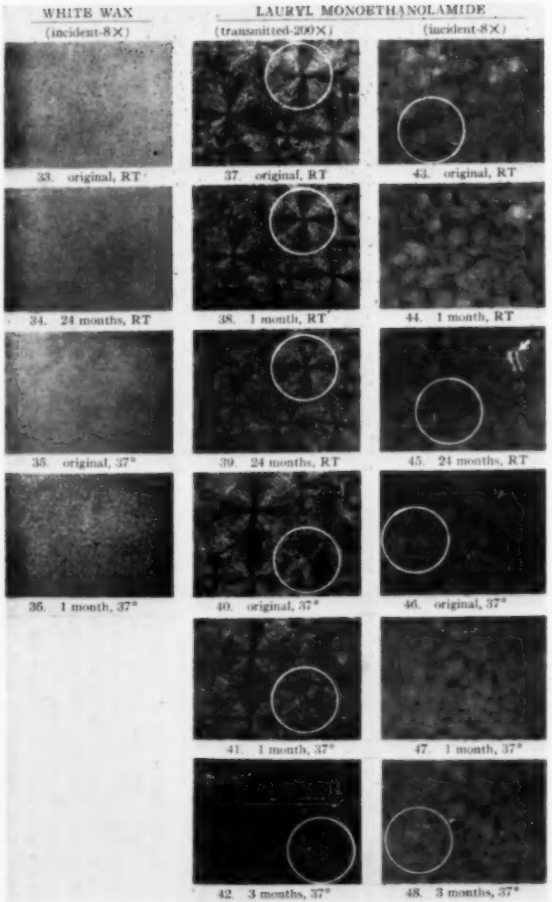


Fig. 3 (*left*).—Photomicrographs showing the crystal structure of white wax and lauryl monoethanolamide.

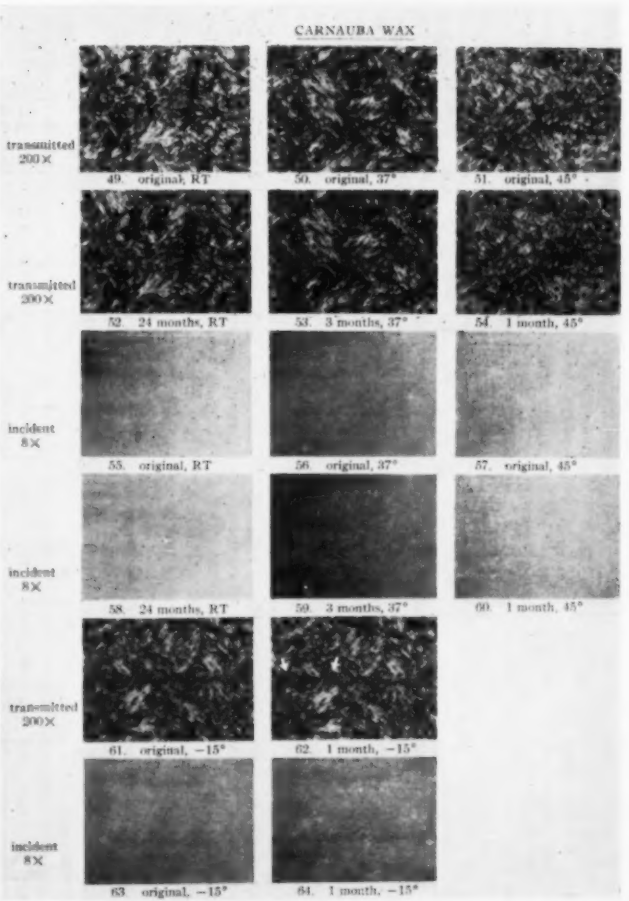


Fig. 4 (*right*).—Photomicrographs showing the crystal structure of carnauba wax.

Note: Some loss of clarity occurred in reproducing the photomicrographs. This is most evident in the photographs taken with incident light, especially Frame 64 (Fig. 4).

heat it gently until melting is complete. Next, spread the molten material into a thin film with a small, warmed glass rod. Then slowly move the rod from one end of the slide to the other until the material begins to solidify. Finally, remove the rod and allow the film of test material to cool. If the slide has been properly prepared, two "wedges" of sample are formed, each tapering from one end of the slide to the center.

At least four such slides were prepared for each material and stored at room temperature for at least two hours before making the original observations. This stabilizing period provided an opportunity for low melting fractions in the sample to crystallize prior to observation.

Original Observations.—At the end of the stabilizing period, each slide was observed under the microscope by transmitted polarized light and scanned at several magnifications. When the most appropriate magnification had been selected, a representative area was photographed. The stage settings employed with each photomicrograph were carefully read and recorded. The type ocular and objective used, bellows length, type lighting system, exposure time, and photometer reading were also recorded. A range in magnification from 50–500× was used.

When the micro observations were completed, the stage, body tube, and light source were replaced with the macro accessories. The slides were placed on the macro stage which was graduated in $^1/_4$ inch units. All macro observations were made by incident light at 8×. After taking the photomicrographs the slides were placed in boxes and stored at either −15°, room temperature, 37°, or 45°.

Subsequent Observations.—Reobservations of the slides were made at one week, and at one, three, six, twelve, and twenty-four months. The areas to be photographed were located by using the stage readings from the original observation and, when necessary, the slides were further centered by comparing the field in the microscope with the original photomicrograph of that slide. The slides were always scanned to detect significant changes that might have occurred in isolated areas. When any such changes were detected, they were photographed and rechecked in later observations.

Observations were discontinued whenever the room temperature slides exhibited a gross change in crystal structure. All slides stored at exaggerated conditions were transferred to room temperature at the end of three months.

RESULTS

It is important to note that the samples were obtained by slowly solidifying the melt. Other methods of sample preparation might have yielded different results. Unless otherwise noted, samples stored at −15° showed no change.

Cetyl Alcohol.—Frames 1–6 (Fig. 1) depict cetyl alcohol magnified 200 times and observed with transmitted light. Corresponding areas within each frame are enclosed by the white circles. Discernable changes occurred after only one month of storage at room temperature (Frame 2). Even more drastic changes occurred at 37° (Frames 5 and 6). Frames 7–12 (Fig. 1) are incident light observations of the same slides but magnified only 8 times. The crystal changes are much less evident at the lower magnification. Although these structural changes are invisible to the unaided eye, the data indicate that cetyl alcohol might not be suitable for uses where crystal integrity is a requirement.

Hydrogenated Soya Oil.—Transmitted light observations of this material are shown in Frames 13–16 (Fig. 1). Although the material has a relatively high melting point of 67°, Frame 14 shows that it undergoes severe crystalline change after storage at room temperature for one month. Some change may be noted even after storage at −15° (Frame 16). As with cetyl alcohol, one would be dubious of using this type of hydrogenated soya oil if crystal integrity is important.

Stearyl Alcohol.—Frames 16–22 (Fig. 2) depict transmitted light observations made after storage at room temperature for twenty-four months, at 37° for three months, and at 45° for three months. Only minor structural changes have occurred under these conditions. Although stearyl alcohol is closely related to the cetyl alcohol shown in Fig. 1, the integrity of its crystal structure is definitely better at the temperatures studied. Frames 23–28 (Fig. 2) show that very little change has occurred in the gross structure of stearyl alcohol. From this study, stearyl alcohol would be considered to have sufficient integrity to permit its evaluation in dosage forms requiring a material of high structural integrity.

White Wax.—White wax is reasonably stable for twenty-four months at room temperature (Frames 29 and 30) (Fig. 2). Frame 32 (Fig. 2) made after storage at 37°, shows several dark areas just below the white arrow. These are thick crystalline areas which have developed. They are dark because light is no longer transmitted through them. The change occurring at 37° is much more clearly shown in Frame 36 (Fig. 3), which is the incident light observation of the same slide. Numerous crystalline areas cover the surface of the slide. These observations indicate that white wax will maintain satisfactory crystal integrity if the storage temperature is approximately 25°. They also indicate that changes occurring at elevated temperatures do not always occur at room temperature within a two-year period.

Lauryl Monoethanolamide.—This material undergoes some structural change when stored for twenty-four months at room temperature or for one month at 37° (Frames 37–41) (Fig. 3). Frame 42 (Fig. 3) shows that a gross change occurs between one and three months at 37° with disappearance of the characteristic structure. The incident light observations of Frames 43–48 (Fig. 3) confirm this, but the degree of change is not so apparent. The white area appearing under the arrow on Frame 44 is the result of damage during handling and should be ignored. These observations indicate that lauryl monoethanolamide can probably be used in dosage forms if structural integrity is not highly critical. Prolonged storage at elevated temperatures should be avoided.

Carnauba Wax.—Frames 49–54 (Fig. 4) show that carnauba wax exhibited very little change in crystal structure when stored at room temperature for twenty-four months, 37° for three months, or 45° for one month. This integrity of the crystal struc-

ture is further exhibited in the incident light observations, Frames 55–60 (Fig. 4). From this data, one would conclude that carnauba wax has properties which may make it valuable for pharmaceutical use. Frames 61–64 (Fig. 4), however, show that carnauba wax cracked when stored at −15°. At high magnification (Frame 62) only a few cracks are visible. Two of these are marked with arrows; the crack on the right being partially covered by the arrow. The clarity of the cracking in Frame 64 made with low magnification shows how the incident light observations often supplement those made with transmitted light. These observations indicate that carnauba wax will maintain its crystal structure at room temperature and at somewhat elevated temperatures. Dosage forms employing it may need to be plasticized when they will be exposed to freezing temperatures.

DISCUSSION

The Panphot camera microscope has several features which we found advantageous. It has a built-in light source, separate oculars for photographic and visual observations, an attached camera with viewing screen, and it can also be used for both macro and micro observations.

The use of Polaroid film permitted rapid development of photomicrographs representing original observations and also eliminated the need for a photographic darkroom. The rapid availability of the photomicrographs permitted the defective ones to be remade prior to storage of the slides at exaggerated temperatures.

Since the area photographed changes inversely with the magnification, total magnification should be just sufficient to permit distinct observation of a characteristic structure. Choice of proper magnification for the micro and macro observations cannot be overemphasized. The micro observations were made with transmitted light to detect minute structural changes. The usual magnification for these observations was 50–200×, but occasionally ran as high as 500×. Magnifications of the photomicrographs used in this communication are sometimes greater than found necessary in practical screening studies. These were employed to enhance clarity in the reproductions of the photomicrographs.

The macro observations were made with incident light to detect gross surface changes which might have an adverse effect on the elegance of pharmaceutical products. Optimum magnification was usually 5–10×. The ability to pick up gross changes at these lower magnifications was clearly shown in the cracked sample of carnauba wax (Fig. 4).

The photomicrographic technique has several features which make it useful: (*A*) thin films of samples are employed to give maximum sensitivity in detection of structural change, (*B*) for reobservations of stored slides, the exact areas observed in the original photomicrographs are used, (*C*) the photomicrographs are permanent and they can be studied whenever desired, and (*D*) the consecutive photomicrographs of a sample facilitate the determination of the approximate rate and severity of structural change. On the other hand, the method has three disadvantages: (*A*) in dosage forms, the structural integrity of the lipid components may be affected by the other components. Therefore, films of single lipids may not always reflect the changes these lipids would undergo when blended with other lipids or pharmaceutical agents, (*B*) the causes of the crystal changes are difficult to interpret, and (*C*) the integrity of the sample following storage cannot be predicted from the original observation.

Crystal transformations in fats and waxes are generally the result of polymorphism, i. e., the ability to exist in more than one crystalline form (1–3). In a polymorphic substance the crystal form present at any given time will depend in part on the heat history of the sample. During storage, if a transition occurs, it is normally toward a more stable crystalline form. Crystal size, melting point, solubility, and other properties may be quite different from those of the original forms. Thus, these new forms, although more stable, may be detrimental to the appearance and desired performance of a manufactured product.

Heterogeneous lipid materials may also show a change in solid:liquid ratio with a change in storage temperature and/or with the passage of time. An increase in solid content occurs as entrapped liquid gradually crystallizes into solid forms, whereas a decrease in solid content occurs when the temperature is elevated above the melting point of low melting fractions in the lipid.

Although the data reported here are limited to several individual lipids, the photomicrographic technique has potential use with lipid mixtures and with all drugs or dosage forms which may undergo crystalline changes.

Among the potential applications for the technique in lipid dosage forms are: (*A*) to determine the maximum amount of a specific lipid raw material which could be used without causing adverse structural effects, (*B*) to determine the effect on lipid structural integrity of introducing a drug into a master formula, and (*C*) to compare the structural integrity of several alternate development forms of a product.

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The Biological Action of Cellular Depressants and Stimulants IV*

The Effect of Phenylurethane on the Onset and the Magnitude of Synchronous Cell Division of *Tetrahymena pyriformis* GL

By WALTER SINGER†, KWAN-HUA LEE, and JOHN J. EILER

Phenylurethane is shown to delay the onset of synchronous division and to decrease the maximum division index in cultures of *Tetrahymena pyriformis* GL synchronized by temperature cycling.

A FIRST APPROACH to understanding how an agent exerts a narcotic action upon cell processes is to measure the effect of the narcotic upon physiological and cytological characteristics of cells. Unicellular microorganisms have often been utilized for such studies. An actively growing population of microorganisms is a heterogeneous mixture of cells representing all stages of a life cycle in which an individual cell divides to form two cells, each of which develops over a span of time into a mature cell which again divides. Recently developed synchronization techniques (1, 2) can produce populations of bacteria, yeast, or protozoa in which most of the cells have been brought into the same phase of the life cycle. Large homogeneous masses of cells are thereby made available for study.

Scherbaum and Zeuthen (3–5) have described a method for synchronizing the ciliate protozoan, *Tetrahymena pyriformis*, by exposure of an actively growing log-phase culture to brief periods of sublethal temperature shocks alternated with exposure to equal periods at a lower temperature optimal for growth. During this temperature cycling, the cells increase in mass but do not divide. When finally returned to, and kept at optimum temperature, the cells grow slightly and begin to divide only after a quite constant lapse of time. After approximately eighty minutes at optimum temperature, about 80 per cent of the cells are seen to be undergoing fission. This essentially simultaneous entry into the phase of division is the evidence that the tetrahymena have been synchronized by the heat treatment. Subsequently, a second and a third synchronous division may be observed.

As a part of a continuing interest in the mechanism of narcotic action, workers in this laboratory have studied (6, 7) the effect of urethane on the growth and respiration of asynchronously growing cultures of *Tetrahymena pyriformis* W and on the oxidative phosphorylation of cell-free preparations (8). The results obtained indicate that the action of urethane to decrease the rate of cell division of tetrahymena is associated with a reduction of an oxygen-dependent growth factor. The system devised for synchronizing tetrahymena offers an opportunity to extend the observations of the effects of members of the urethane series to (*a*) tetrahymena being synchronized by temperature cycling and to (*b*) the resultant synchronized cells. Such a study deals, in the first instance, with cells that are growing but not able to divide and, in the second, with cells that are all in the same phase of their life cycle and soon destined to enter simultaneously into the process of cell fission. Accordingly, this investigation was concerned with the effects of phenylurethane, a potent narcotic, upon significant biological parameters related to growth and cell division in cultures of tetrahymena treated to produce synchronous cell division. This is a report of the action of phenylurethane to delay the onset of synchronous division and to decrease its magnitude. Effects of this agent upon cell volume increase and upon the synthesis of protein and desoxyribonucleic acid during and after heat treatment are reported separately (9).

MATERIALS AND METHODS

Culture Techniques.—Axenic stock cultures of *Tetrahymena pyriformis* GL (obtained through the courtesy of G. W. Kidder) were maintained in slanted test tubes at 28° in 1.8% proteose-peptone (Difco) plus 0.2% yeast extract (Difco). Cells (0.6 to 15 × 10^6 in number) from a forty-eight hour old stock culture were inoculated into 400 ml. of sterile culture medium in a 2-L. Erlenmeyer flask

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† Fellow of the American Foundation for Pharmaceutical Education, 1955–1956, and Smith, Kline and French Fellow, 1956.

and allowed to multiply for twelve to twenty-four hours to provide a sufficient quantity of organisms for each experiment.

Synchronization Procedure.—The synchronization procedure was patterned after that developed by Scherbaum and Zeuthen (2–5). Twenty-five milliliter suspensions of logarithmically growing cultures in proteose-peptone and yeast extract medium (0.2 to 2.4 × 10^5 cells per ml.) were aseptically pipeted into sterile 300-ml. Erlenmeyer flasks, usually six in number. The flasks were suspended in a water bath fitted with controls (10) which could be set to alternate the water temperature between two levels. The cells were exposed to half-hour periods at 34° alternated with half-hour periods at 28.2°. It required eight minutes to raise the bath temperature from 28.2 to 34° and twelve minutes to lower the temperature again. The eight minutes were included in the half-hour at 34°; the twelve minutes were included in the half-hour at 28.2°. After the eighth period at 34°, the bath temperature was allowed to drop to 28.2° and was then kept constant at this level. The flasks were shaken throughout the treatment. With this procedure, a good first synchronous division was always obtained and a second was sometimes seen. A third synchronous division was not observed to occur.

Cell Counting.—One-milliliter samples were pipeted from test cultures at appropriate intervals. The cells were killed by the addition of 0.3 ml. of formalin. The number of cells per ml. of sample was determined using a Sedgewick-Rafter counting chamber and a Whipple micrometer (6, 11, 12). A second count of only the number of dividing cells was also made for each sample. Any cell showing any degree of cytoplasmic fission was considered to be a dividing cell. A 5-power ocular and 16-mm. objective were used. Photomicrographs were taken of key samples.

Reagents.—Phenylurethane (Eastman) C_6H_5-$NH \cdot COOC_2H_4$), recrystallized once from petroleum ether, was dissolved in 95% ethanol to prepare stock solutions such that 0.05 ml. added to 25 ml. of cell suspension would provide the desired final drug concentration.

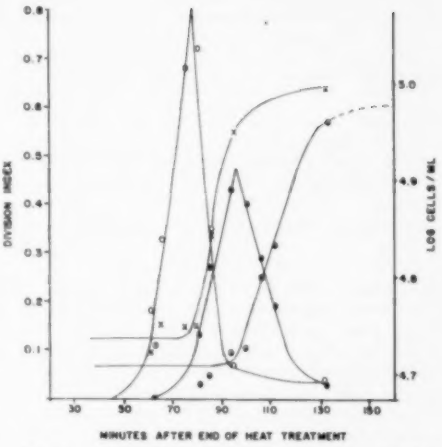

Fig. 1.—The effect of phenylurethane on division index and on population density: 6 × 10^{-4} *M* phenylurethane added before heat treatment. Division index, ○ = control, ● = drugged. Population density, X = control, ⊗ = drugged.

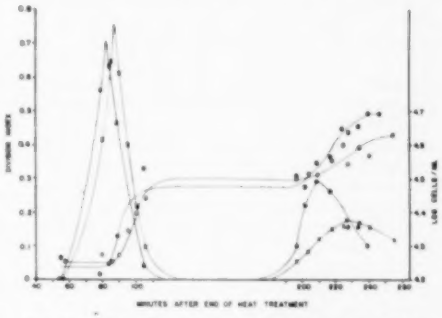

Fig. 2.—The effect of phenylurethane on division index and on population density: 6 × 10^{-4} *M* phenylurethane added after heat treatment. Division index, ● = control, X = drugged. Population density, ⊗ = control, ○ = drugged.

RESULTS AND DISCUSSION

One measure of the degree of synchronization is the division index (4) which is defined as the ratio of the number of dividing cells to the total number of cells at a given instant. The maximum division index, i. e., the highest such ratio found during the time interval through which the synchronous division takes place, is a most useful criterion of synchronization. Its magnitude and the time at which it occurs can be readily accurately determined by a graphical method using values for division indexes from the several samples taken at intervals during a synchronous division outburst (see Figs. 1 and 2). Under the experimental conditions used, the value of the maximum division index and the length of the lag period (the interval between the end of temperature cycling and the occurrence of the maximum division index) were found to be quite reproducible for first synchronous division.

The effect of phenylurethane on these parameters of synchronous division and upon increases in cell number was studied under two general conditions, *viz.*, when the drug was added just before starting the heat treatment and when it was added approximately fifteen minutes after the final 34° level. In either case, 0.05 ml. of the drug in 95% ethanol was added aseptically to 25 ml. of cell suspension to yield a final concentration of 6 × 10^{-4} *M* with respect to phenylurethane. This concentration has been shown by conventional turbidity measurements to inhibit reversibly the total cell mass increase (by 50%) in asynchronous cultures of tetrahymena; it also decreases the rate of cell multiplication in these cultures as measured by cell count (unpublished data).

The Effect of Phenylurethane on First Synchronous Division: 6 × 10^{-4} *M* Phenylurethane Added Before Heat Treatment.—The effects of phenylurethane on the first synchronous division of cells which were synchronized by heat treatment in the presence of the drug are shown by data in

TABLE I.—THE EFFECT OF PHENYLURETHANE ON FIRST SYNCHRONOUS DIVISION

	Max. Index	Time[a]	Cells/ml. × 10^{-4} Log Phase	Prediv.	Postdiv.	Post-/Pre-
Controls[b]	0.77 ± 0.01	80 ± 0.7	47.7	54.4	95.1	1.7 ± .03
Drugged[b]	0.42 ± 0.02	100 ± 0.9	47.7	53.3	79.9	1.5 ± .04
Controls[c]	0.69 ± 0.02	83 ± 1.6	...	...	...	...
Drugged[c]	0.61 ± 0.03	89 ± 1.8	...	...	...	...

[a] The number of minutes between the end of the heat treatment and the attainment of the maximum division index.
[b] Average values for 11 experiments in which 6×10^{-4} *M* phenylurethane was added before heat treatment.
[c] Average values for 11 experiments in which 6×10^{-4} *M* phenylurethane was added after heat treatment.

The standard errors shown here were calculated from the formula: $S.E._{\bar{x}} = \pm\sqrt{\frac{\Sigma(X - \bar{X})^2}{n(n-1)}}$

Table I and in Fig. 1. It should also be brought out here that, during the heat treatment, the control cells almost tripled in size and in protein content relative to log-phase cells, and that the drugged cells increased only to about three-fourths of the size of the control cells (9). One effect is a decrease in the magnitude of the maximum division index; the average value for drugged cells was 54.5% of that for the controls. A second is a twenty-minute increase in the time interval between the end of the heat treatment and the attainment of the maximum division index. Figure 1 is a graphical presentation of these effects as observed in a typical experiment. It should also be noted that the delay in reaching the maximum division index was accompanied by a delay in the onset of division. The delay in onset, although clearly evident (Fig. 1), was not amenable to quantitative treatment because it was difficult to pinpoint the time at which the first cytoplasmic furrowing occurred.

A third effect of the drug, when added prior to heat treatment, is a decrease in the number of cells resulting from the first synchronous division. In Table I, the population densities of control and of drugged cultures are compared. The predivision density is the average number of cells per ml. in the three or four samples taken before peak division index; this represents the cell population at the end of the heat treatment. Predivision population densities of drugged cultures did not differ significantly from those of the controls. Each postdivision density was obtained from a single sample collected when the first synchronous division outburst was over. Table I shows that the postdivision population density of drugged cultures averaged 84% of that of the controls. The 1.5-fold increase in drugged cell count during the synchronous division is significantly lower ($p = 0.01$) than the 1.7-fold increase in control cell count.

A fourth drug effect is an increase in the time needed to complete the first synchronous division outburst once it has started. In Fig. 1 it can be seen that the rate of change in division indexes was less rapid in the drugged culture than in the control. Also it is apparent in Fig. 1 that the cell population in the drugged culture increased less rapidly than in the control.

The lesser slopes of these temporal changes in division indexes and in population increases suggested that phenylurethane might be adversely affecting the induced phasing of the tetrahymena. Evidence for this point of view was obtained from a differential count of fission stages. This count was made from photomicrographs taken of cell suspensions which were just before or just after peak division. Five categories of cells were set up, namely, (*a*) not divided, (*b*) starting to divide, (*c*) half-way divided, (*d*) nearly split apart, and (*e*) daughter cells. The decision to place a cell into a specific category had to be based upon a close scrutiny of the outline of the fixed cell followed by an estimation of how far along in the fission process the cell had progressed. Some inaccuracy must necessarily result from attempting to divide into discontinuous groups a continuous action such as cell fission.

The data are summarized in Fig. 3. For the control culture, the finding of the definite high per cent peak at progressively later fission stages as the time of maximum division index was approached and passed, shows that a large group of these cells began to divide at the same time and continued to pass as a group through the entire fission process. This is the picture to be expected for good synchrony.

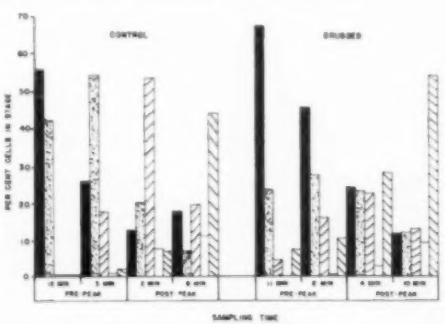

Fig. 3.—The effect of phenylurethane on the distribution of fission stages of Tetrahymena during first synchronous division: 6×10^{-4} *M* phenylurethane added before heat treatment. Approximately 2,500 drugged and 2,500 control cells were cataloged. ■ = not divided; ▨ = starting to divide; ▨ = half-way divided; □ = nearly split apart; ▨ = daughter cells.

The drugged cells did not show this formation of a well-defined group. Large groups were found only at two times, i. e. at eleven minutes pre-peak when most of the cells had not yet begun to divide and again at ten minutes postpeak when daughter cells were in the majority. Near the time of the maximum division index, especially shortly postpeak, the cells were about evenly proportioned among four stages. This is in sharp contrast to the findings in the control culture and indicates that phenylurethane had interfered with the phasing of the cells.

Each of the effects on the parameters of first syn-

chronous division which are observed when phenylurethane is added before heat treatment can be attributed to inhibitory actions of the drug on a cellular system or systems concerned with division. The delayed onset of division is direct evidence that phenylurethane either slows formation of material essential to initiation of division or else prevents for some time the action of the material once formed. The out-phasing of the cells manifested as a decreased rate of change of division indexes and of population can arise from a randomized entry into the division phase due to individual variation in cell susceptibility to phenylurethane inhibition. Some cells are affected by the drug to such an extent that they fail to divide during the synchronous division outburst. There would appear to be a relationship between the failure of a proportion of the drugged cells to divide during the first synchronous division and the observed lowered value for the maximum division index of the drugged cells. The drugged cell population was found to average 84% of that of the control cells after completion of the division outbursts (Table I). From the maximum division indexes (Table I), one can calculate that the ratio of the number of drugged cells to control cells after synchronous division would be 1.42/1.77, i. e., that the drugged cell population would be 80% of that of the control cells. The correspondence between these figures indicates that the lowered division index reflects an action of phenylurethane to prevent the division of a number of cells. Possibly the out-phasing of the cells also contributes to lowering the maximum division index.

Data in Table I provide evidence that cells may reach a stage in the fission process which is insensitive to inhibition by phenylurethane. The small increase in cell numbers which occurred during the heat treatment is taken to indicate that temperature cycling did not stop the division of cells which had already begun to divide (5). Since the drugged cell and the control cell populations increased equally during heat treatment, apparently phenylurethane also did not stop division once it had actually started.

Effect of Phenylurethane on First and Second Synchronous Division: 6×10^{-4} *M* Phenylurethane Added After Heat Treatment.—When phenylurethane was added to the culture medium of synchronized cells at approximately fifteen minutes after the end of the final 34° heat treatment, two effects on the division index of the first synchronous division were noted. These were qualitatively but not quantitatively the same as those found when the phenylurethane was added prior to synchronization. The division index maximum for the drugged cells was lower and it was reached later. However, as shown by the data in Table I and Fig. 2, the effect of the phenylurethane added after synchronization was less marked than that produced by adding it prior to synchronization. Although the division index maximum was lowered in 9 out of 11 experiments, the effect was pronounced in only one experiment. In five experiments, the drugged cell maximum index was between 80 and 90% of that of the controls; in three others it was more than 90% of the control value and, in two experiments, drugged cell division index maxima were slightly higher than the control values. The average drugged cell maximum division index was 88.4% of the control value. In these same experiments, the drugged cells reached maximum division index, on the average, six minutes later than did the control cells. The delay, observed in 10 of the 11 experiments, is statistically significant ($p = 0.05$).

When phenylurethane was added after heat treatment, it showed little ability to prevent cells from dividing during first synchronous division. Post-division population data are not available because the taking of samples was terminated in a number of these experiments before the drugged cell synchronous division was completely over. However, when data from individual experiments are plotted as in Fig. 2, it can be seen that exposure to phenylurethane only during the lag period did not consistently decrease the number of cells present at the end of first synchronous division. This is in contrast to the decrease produced when the drug was added before heat treatment.

In two experiments in which phenylurethane was added after heat treatment, data for a second synchronous division were obtained. Figure 2 includes these data for one experiment. The control cells were no longer well phased. This was shown by the lower maximum division index (0.28), by the increased spread of the division index curve along the time axis, and by the lesser slope of the population density curve when compared to the similar features of the first synchronous division. The phenylurethane, which had been in contact with the cells since shortly after the conclusion of the heat treatment (about three and one-half hours), exaggerated each of these changes. When compared with the controls, the maximum division index of the drugged cells was lower (0.18), the spread of the division index curve along the time axis is further widened, and the slope of the population density curve is clearly lessened. In addition, the drugged cell maximum index occurred twenty-one minutes later than did that of the controls; the delay was only five minutes during the first synchronous division. It is also evident that at the end of the second synchronous division the drugged cell population density was less than that of the controls.

Thus, phenylurethane added after the synchronizing heat treatment is completed exerts more pronounced effects upon the second synchronous division than upon the first. In fact, the effects upon second synchronous division are quantitatively quite similar to those which phenylurethane added before heat treatment has upon the subsequent first synchronous division.

Synthesis of cellular material is a characteristic common both to the period of heat treatment and to the period between the first and the second synchronous division. There appears to be relatively little synthesis of protein during the first lag period (9, 13); DNA may (14) or may not (9) be synthesized. Phenylurethane shows its greatest effect on synchronous divisions which follow contact of the drug with tetrahymena during a time of active growth. The drug does not prevent cell fission once it has started. The evidence is, therefore, that the effects of phenylurethane reported herein are a consequence of its overall inhibition of cellular synthesis which slows or prevents formation of a material essential to initiation of fission.

SUMMARY

1. A narcotic concentration of phenylurethane is shown to delay the onset of synchronous division and to reduce the maximum division index in cultures of *Tetrahymena pyriformis* GL synchronized by temperature cycling.

2. Phenylurethane effects are more pronounced when the drug is present during the temperature cycling than when the drug is added after the cycling.

3. The delay in onset of division is attributed to a decreased rate of production of material necessary for cell division. Drug-induced randomization of the entry of cells into division is a reflection of the varied susceptibility of the cells. The lowered maximum division index results from failure of some of the more susceptible cells to divide.

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The Development of a Liquid Antihistaminic Preparation With Sustained Release Properties*

By HARRY A. SMITH†, ROBERT V. EVANSON, and GLEN J. SPERANDIO

The relative capacity of several cation exchange resins to adsorb methapyrilene has been determined by an *in vitro* method. It was demonstrated that methapyrilene adsorbed on sulfonic acid cation exchange resin possessed pharmacological activity approximating that of a solution of methapyrilene hydrochloride as measured by the protection against histamine-induced asthma in the intact guinea pig. It is suggested that the use of ion exchange resins as carriers of drugs in liquid dosage forms may be useful in sustained release preparations.

THE COMMERCIAL USES of ion exchange resins have steadily increased since the first synthetic ion exchange resin was prepared by Adams and Holmes in 1935 (1), and the number of publications per year pertaining to ion exchange almost doubled from 1950 to 1955 (2). The use of ion exchange materials in medicine is as old as the medical profession itself (3), a good example being the use of kaolin and similar substances as enteric adsorbents of toxins. The use of synthetic ion exchange resins as medicinal agents has been reported in the medical literature (4–6).

The literature (7–13) indicates that the principle of sustained release of a drug from its resin-adsorbate might well be applied to orally administered suspensions of the drug. The chief objective of this project was to develop a liquid antihistaminic with sustained release properties.

Although there are available long acting antihistamines, the availability of a sustained release antihistamine in a liquid formulation would be of value in cases where solid medication cannot be taken. Moreover, the demonstration of the feasibility of using cation exchange resins to obtain a controlled, sustained release of drugs in a liquid form was a major consideration.

EXPERIMENTAL

Capacity Studies.—Methapyrilene was chosen as the antihistamine for study and the following cation exchange resins were screened for their capacity to adsorb methapyrilene using the batch technique: Dowex 50[1] resins with 4, 8, and 12% cross-linkage, each with 50–100, 100–200, and 200–400-mesh ranges; and Amberlite IRC 50[2] resins with a 4–6% cross-linkage and 16–50, 100–200-mesh ranges, and 90% passing through a 200-mesh sieve were used in this work. Cross-linkage refers to the internal porosity of the molecular resin structure and is determined by the amount of divinylbenzene added during the styrene-divinylbenzene copolymerization. All of the resins were used in the acid

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[1] Dowex 50 is the trade name of Dow Chemical Co. for their series of sulfonic acid cation exchange resins.
[2] Amberlite IRC is the trade name of Rohm and Haas Co. for their series of carboxylic acid cation exchange resins.

form. For a comparative study the resins were employed on a dry weight basis, since the various resins adsorb varying percentages of water. The moisture content of each resin was determined with the Cenco moisture balance just prior to each capacity determination.

The batch technique was employed to determine the relative capacity of the cation exchange resins to adsorb methapyrilene. One-gram samples of the resins were mixed with 50 ml. of a 0.1*M* solution of methapyrilene hydrochloride and agitated until apparent equilibrium was reached. This was determined by analyzing for the unadsorbed methapyrilene spectrophotometrically, using the method of Martin and Harrisson (14). When there was no significant change in the amount of unadsorbed methapyrilene, apparent equilibrium was assumed to be established. The amount of methapyrilene adsorbed was calculated as the difference between the amount of methapyrilene hydrochloride in the original solutions and the amount of unadsorbed methapyrilene hydrochloride.

Preliminary experiments were conducted to determine the effect of the concentration of methapyrilene hydrochloride on the amount of methapyrilene adsorbed by a sulfonic acid cation exchange resin with a 4% cross-linkage and a 50–100-mesh range. The results are shown in Fig. 1. A study of these data led to the selection of a 0.1*M* solution of methapyrilene hydrochloride for further capacity studies since that was the lowest concentration to permit differentiation among the several resins for their capacity to adsorb methapyrilene.

A comparative study was made of the capacity of several sulfonic acid cation exchange resins with various degrees of cross-linkage and particle sizes to adsorb methapyrilene from 50 ml. of a 0.1*M* solution. The data are shown in Table I.

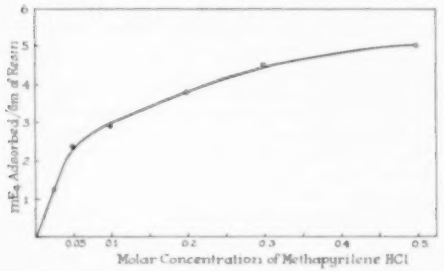

Fig. 1.—The effect of concentration on the amount of methapyrilene adsorbed from solution by Dowex 50, 4% cross-linkage 50–100 mesh.

TABLE I.—THE EFFECT OF CROSS-LINKAGE AND PARTICLE SIZE ON THE ADSORPTION OF METHAPYRILENE BY SULFONIC ACID RESINS

Mesh Range of Resin	meq. of Drug Adsorbed/Gm. of Resin at Apparent Equilibrium		
	4% Cross-Linkage[a]	8% Cross-Linkage[a]	12% Cross-Linkage[a]
50–100	2.71	2.66	2.30
100–200	2.64	2.55	2.31
200–400	2.71	2.65	2.08

[a] Values are the average of three samples.

The results show that adsorption of methapyrilene decreases with an increase in the degree of cross-linkage of the resins. Also particle size has no apparent effect on the amount of adsorption of the drug at apparent equilibrium. However, it was observed that with the 12% cross-linkage, the rate of adsorption was greater with the smaller particle sizes.

Similar experiments were conducted with the carboxylic acid cation exchange resins, except that the adsorption process was permitted to proceed for only three hours for a comparative study. The quantities of methapyrilene adsorbed, expressed in milliequivalents per gram of dry resin, were 1.37 for the 16–50 mesh, 1.37 for the 100–200 mesh and 1.65 for the 200–400 mesh.

It was found that the sulfonic acid resins exhibited a greater relative capacity for adsorbing methapyrilene than the carboxylic acid resins under the conditions of these experiments. However, all of the resins adsorbed a sufficient quantity of methapyrilene per gram of dry resin to permit their use as medicinal carriers.

Rate of Release Studies.—Experiments were conducted to determine the rate of release of methapyrilene from cation exchange resins under uniform conditions approximating those of the upper gastrointestinal tract. The rate of release of the drug from the resin-adsorbate was determined by measuring the amount of methapyrilene released into 100 ml. of 0.1*N* hydrochloric acid at different time intervals. A sample of the resin-adsorbate equivalent to 100 mg. of methapyrilene hydrochloride was used. It was mixed with the acid in a sintered-glass-bottomed funnel with a capacity of approximately 190 ml. and the resin-adsorbate was kept well suspended by an electric stirrer, with its rotational speed controlled by a variac. At the end of fifteen minutes, the exchange medium was filtered with the aid of a water aspirator, and the filtrate was analyzed spectrophotometrically for the amount of methapyrilene which had been released into it. Fresh exchange solution was added to the resin adsorbate and the process was repeated for the desired number of exchange periods.

Experiments with a carboxylic acid resin-adsorbate indicated that this adsorbate had a very rapid rate of release of methapyrilene into 0.1 *N* hydrochloric acid and is largely converted to the hydrogen form of the resin within fifteen minutes. Since a slower and more prolonged release of the drug was desired, the sulfonic acid type resins appear to be the carriers of choice for methapyrilene.

A series of experiments was conducted to determine the effect of particle size and degree of cross-linkage on the rate of release of methapyrilene into 0.1 *N* hydrochloric acid from adsorbates prepared from sulfonic acid cation exchange resins. The method described previously was used except that after four hours (16 fifteen-minute exchange periods), the time intervals were extended to one hour periods, and the process was continued for a total of twelve hours. The longer periods were necessary after it was found that beyond four hours the amount of methapyrilene released in a fifteen-minute period was less than 1%. The data from these experiments are presented in Figs. 2–4.

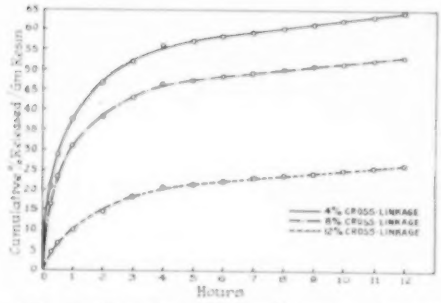

Fig. 2.—The effect of cross-linkage on the rate of release of methapyrilene from Dowex 50, 50–100 mesh into 0.1 *N* HCl.

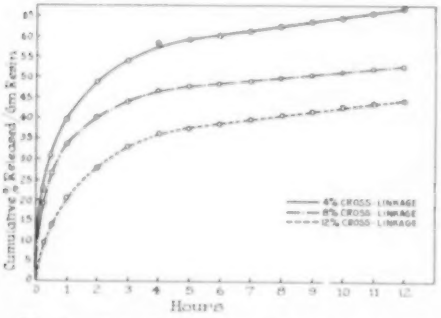

Fig. 3.—The effect of cross-linkage on the rate of release of methapyrilene from Dowex 50, 100–200 mesh into 0.1 *N* HCl.

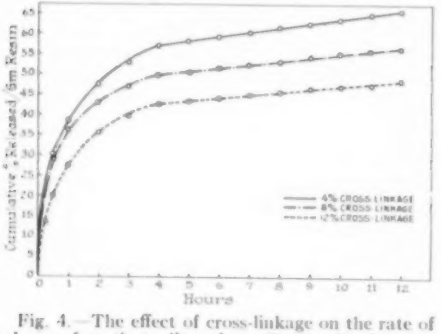

Fig. 4.—The effect of cross-linkage on the rate of release of methapyrilene from Dowex 50, 200–400 mesh into 0.1 *N* HCl.

Examination of these data shows that the rate of release of the drug was inversely related to the degree of cross-linkage of the resins. Also, it reveals that there was a greater difference in the rate of release between 12 and 8% cross-linked resins than between 8 and 4% cross-linked resins. This indicates a critical resin porosity between 8 and 12% cross-linkage, above which the rate of exchange is greatly reduced. Also, it appears that, with a decrease in particle size, the effect of cross-linkage becomes relatively less.

The effect of particle size on the rate of release was less evident with resins of cross-linkage below 8%. However, with the 8 and 12% cross-linked resins, it was apparent that the rate of release was inversely related to the particle size.

Consideration of the rate of release, the extent of release over a twelve-hour period, the palatability, and suspendability of the various resins led to the selection of a resin with 4% cross-linkage and a 200–400-mesh range for formulation and *in vivo* studies.

Formulation Studies.—Several nonionic suspending agents were used individually and in combinations in an attempt to formulate a stable and palatable suspension of the selected resin-adsorbate. These experiments resulted in the following formula:

Resin-adsorbate	*q. s.*
Vanillin, 5% in alcohol	1%
Span 85[3]	0.2%
Methylcellulose 1,500 c. p. s. 1% solution	
Simple syrup	*aa q.s.* ad 100%

This formulation resulted in an excellent product with no noticeable sedimentation after standing for thirty days. The product was palatable except for a slight grittiness, which is inherent in the resin particles. Span 85 was included in this product to minimize foaming which occurred when it was agitated.

***In Vivo* Studies.**—The *in vivo* studies involved a comparison of the protection against histamine-induced asthma in the intact guinea pig provided by a solution of methapyrilene hydrochloride and a suspension of the resin-adsorbate administered orally. The method employed was a modification of the one used by Lee, *et al.* (15). It is based on the principle that inhalation of a histamine aerosol by guinea pigs induces bronchial asthma, which can be lessened in severity by an antihistaminic agent.

Guinea pigs weighing from 500–800 Gm. were used. They were fasted for twelve hours prior to each experiment. The histamine aerosol was produced by nebulizing with a De Vilbiss No. 40 atomizer a 1:80 solution of histamine diphosphate with seventy mm. of Hg air pressure. The time required for each untreated animal to evince prominent asthmatic symptoms, as manifested by laborious breathing and a prominent gasp, was recorded as the control time in seconds with an electric timer. Control tests were conducted with each animal before each experiment, and animals with a control time much greater than two minutes were not used. The animals were permitted to rest one hour after the control tests, then were dosed with the desired compound by stomach intubation.

The individual animals were tested hourly, with only two exceptions, by exposing them to the histamine aerosol. The exceptions occurred during one experiment with animals number 3 and 9 which were tested at one-half hour intervals after eight hours. The criterion for measurable protection was arbitrarily selected as an exposure of twice the control time before the appearance of prominent asthmatic symptoms. This criterion corresponds to the one used by Feinberg, *et al.* (16). This method allowed a direct comparison

[3] Span 85 is a trade name of the Atlas Powder Co. for their brand of sorbitan trioleate.

of the protection provided by orally administered methapyrilene and the resin-adsorbed methapyrilene.

Four experiments were conducted with six guinea pigs. Three animals were given the resin-adsorbate and three were given a solution of methapyrilene hydrochloride. The animals were crossed over at two-day intervals so that all animals received both the resin adsorbate and the methapyrilene twice.

Table II shows the results of these experiments.

TABLE II.—PROTECTION AGAINST HISTAMINE AEROSOL DYSPNEA IN GUINEA PIGS BY METHAPYRILENE AND RESIN-ADSORBED METHAPYRILENE

Methapyrilene —Controls—		Resin-Adsorbed Methapyrilene	
Animal No.	Hours	Animal No.	Hours
2	5	1	4
4	6	3	6
6	7	9	6
1	5	2	8
3	8	4	4
9	4	6	5
2	5	1	..
4	3	3	9.5
6	5	9	8.5
1	..	2	8
3	6	4	7
9	7	6	6
Average	5.5	..	6.5

The animals dosed with the resin-adsorbed drug had an average duration of protection of six and one-half hours, whereas the control animals had a duration of protection of five and one-half hours. The difference, however, was not significant as indicated by the Fisher "t" test ($t_{80} = 1.46$; $0.2 > p > 0.1$). The value of 1.46 for "t" indicated a probability level less than 0.2, but greater than 0.1. Although the results seemed to indicate a longer duration of protection with the resin-adsorbed drug, the difference was not statistically significant in light of the large variation in the animal responses. The experiments demonstrated only that the resin-adsorbed drug possessed pharmacological activity at least equal, if not superior, to the solution of methapyrilene hydrochloride.

DISCUSSION

The *in vitro* studies indicated that while the initial release of methapyrilene from its resin-adsorbate is relatively rapid, the rate decreases with time. The *in vivo* studies indicated that this initial release of drug is sufficiently rapid to provide a therapeutic effect in the guinea pig within thirty minutes or less.

Also, the *in vitro* experiments showed that without the addition of fresh exchange medium, the exchange process soon reached apparent equilibrium. When this condition is obtained in a biological system, it is to be expected that the rate of release of the drug will depend essentially on its rate of absorption through the intestinal villi. After a certain point in the process, the rate of absorption through the intestinal villi probably will exceed the rate of release of the drug from its adsorbate, at which time the blood level of the drug may begin to fall.

Since only a portion of the drug is released during the early stage of the process, the blood level should not exceed that necessary for therapeutic effects by such a great margin as that which occurs when all of a drug is immediately available for absorption. Thus, undesirable side effects may be diminished or eliminated. Another advantage of the use of ion exchange resins for medicinal carriers is the elimination of the variations in the blood levels of drugs which often occur with repeated doses of conventional dosage forms. This statement is based on the fact that the rate of release of the drug from its resin-adsorbate is continuous over a long period of time and decreases uniformly.

Further work is indicated to prove conclusively the above hypothesis. The use of a drug tagged with a radioactive isotope seems suited for such work because this technique permits the direct measurement of the drug in the body fluids and tissues.

SUMMARY

1. The relative capacity of several cation exchange resins to adsorb methapyrilene was determined.

2. The rate of release of methapyrilene from its resin-adsorbates was studied *in vitro*. The carboxylic acid resin-adsorbate exhibited a very high exchange rate in an acid medium (0.1 *N* HCl). The sulfonic acid cation exchange resins are applicable for a controlled, sustained release of ionic drugs, and the rate of release may be controlled by the proper selection of the cross-linkage and particle size of the resin.

3. A stable suspension of a selected resin-adsorbate was formulated without materially affecting the rate of release of methapyrilene.

4. Methapyrilene adsorbed on a sulfonic acid cation exchange resin possessed pharmacological activity at least equal, if not superior, to a solution of methapyrilene hydrochloride, as measured by the protection against histamine-induced asthma in the intact guinea pig.

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The Influence of Some Suspending Agents on the Release of a Soluble Medicament from Solution*

By G. D. REDMAN, J. E. CHRISTIAN, and GLEN J. SPERANDIO

A method for comparing the influence of suspending media on the dialysis of a soluble compound is described. Different suspending agents affected the rate at which the iodide ion dialyzes from solution, indicating that the nature of the agent is a variable which influences the release of the compound. It is demonstrated that the use of selected suspending agents makes possible the modification of release rates of certain soluble drugs from solution.

IN RECENT YEARS a number of new approaches to the preparation of dosage forms for oral administration have been made which are intended to produce a sustained level of drug action. Most of the work thus far has been in the development of tableted or encapsulated medicinal substances, although liquid preparations intended for oral administration which would have the ability to release incorporated active ingredients over an extended period of time may find application in the areas of geriatric, pediatric, and psychiatric medicine. Therefore, this project was initiated in an attempt to find a way to prepare liquid medication with sustained release properties. It was thought that one possible way of influencing drug release from solutions might be to add certain substances which might delay the process of dialysis; and some commonly used suspending agents were chosen for initial investigation.

A number of workers have utilized radioisotopes to study the release of substances from pharmaceutical preparations. Johnston, *et al.* (1), studied the absorption of $Na^{24}Cl$ from the ointments by the intact skin of rats. Cyr, *et al.* (2), measured the absorption of NaI^{131} which had been incorporated into ointment bases through the skin of rats. Lux, *et al.* (3), studied the iodide ion permeability of living frog membranes using sodium radioiodide.

An *in vitro* procedure for the determination of the release of anions and cations from emulsified ointment bases was reported by Stark, *et al.* (4). Trace quantities of NaI^{131} and of $Hg^{203}(NO_3)_2$ were incorporated independently into emulsified ointment bases and the dialysis of the ions in a dialysis apparatus especially adapted for the detection of radioactivity present in the dialysate was used.

Other workers have employed dialysis procedures to study the dispersion of a wide variety of substances (5–10).

This investigation utilized iodine^{131}-labeled iodide ion to study the effect of different suspending agents on the dialysis of the iodide ion through a semipermeable membrane.[1] The effect of different concentrations of two selected suspending agents on dialysis was also determined.

EXPERIMENTAL

The composition and suppliers of the individual suspending agents which were chosen for study are shown in Table I.

TABLE I.—COMPOSITION AND SOURCE OF SELECTED SUSPENDING AGENTS

Suspending Agent	Composition	Supplier
Attagel 20	Activated attapulgite clay	Minerals & Chemical Corp. of America, Menlo Park, N. J.
Cato 8	Cationic corn starch derivative	National Starch Products Inc., Plainfield, N. J.
Jaguar gum	Galactomannan from endosperm of guar seed	Stein Hall & Co., Inc., New York, N. Y.
Locust bean gum	Galactomannan from endosperm of locust bean seed	Stein Hall & Co., Inc., New York, N. Y.
Methocel 400	Methylcellulose U. S. P., a methyl ether of cellulose	Dow Chemical Co., Midland, Mich.
Pectin N. F.	Complex hydrophilic colloidal carbohydrate, chiefly partially methoxylated polygalacturonic acids	S. B. Penick & Co., New York, N. Y.
Ramalin G	Amylopectin	Stein Hall & Co., Inc., New York, N. Y.
Superlose	Amylose	Stein Hall & Co., Inc., New York, N. Y.
Tragacanth U. S. P.	Complex hydrophilic carbohydrate composed of glucuronic acid, arabinose, and methoxylated acids	S. B. Penick & Co., New York, N. Y.
Veegum	Magnesium aluminum silicate	R. T. Vanderbilt Co., New York, N. Y.

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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August, 1959.

[1] Type SS seamless, regenerated cellulose dialysis tubing; average pore radius 24 Å. Visking Corp., Chicago, Ill.

One per cent dispersions of each suspending agent were prepared in 200-ml. quantities. Two grams of the agent were dispersed in approximately 100 ml. of U. S. P. phosphate buffer solution (pH 7.0) to form a slurry. To the slurry 4 ml. of a 0.5% solution of potassium iodide U. S. P. and 0.2 Gm. of methylparaben U. S. P. were added, and the volume was brought to 200 ml. with additional buffer solution. The slurry was quantitatively transferred to a Liquidizer[2] and mixed at low speed. A 500-λ quantity of solution containing approximately 4 μc. of iodine^{131} was added to a 35-ml. aliquot of the suspending media. The added activity imparted approximately 3,000 counts per minute per 5 ml. of the sample as determined by the counting procedure described below.

A dialysis apparatus described by Stark (4) was modified for use in this study. A glass cylinder used as the sample holder was firmly secured to the remainder of the dialysis apparatus by stretching rubber bands between glass projections fused to the apparatus. A rubber washer, cut from 3/4 inch stock, was placed around the base of the sample holder and positioned flush with its ground-glass surface to prevent the glass from cutting the membrane. A thin film of silicone lubricant was evenly spread on the ground-glass surface of the sample holder to reduce the possibility of sample leakage.

The membrane was attached to the dialysis apparatus by the procedure described by Stark (4) and normal saline solution was used as the dialysate. The apparatus was placed in a constant temperature bath to maintain the temperature of the dialysate at 37° ± 1°.

Five-milliliter samples, accurately measured and checked by weighing, of the suspending media prepared for test were placed in the sample holder of the dialysis apparatus. The dialysate was circulated through a closed system past the surface of the semipermeable membrane and around a counting tube to detect the migrating ions. A gamma-sensitive bismuth cathode counting tube was used in conjunction with a Model 1620 analytical count rate meter.[3] The per cent probable error of a single reading after equilibrium was reached, based on a fifty-second time constant and an expected count, was 0.85. An Esterline-Angus Model AW milliammeter graphic recorder[4] was used to provide a continuous recording of the dialysis of the iodide ion.

Prior to each determination a 5-ml. quantity of the radiolabeled suspending media, accurately determined and equal to that used in the release studies, was placed in the lumen of the counting chamber and dispersed in the dialysate. The activity of this sample corrected for background represented 100% activity of the sample and was used as the standard to express the percentage release of the iodide ion.

RESULTS

The influence of equal concentrations of suspending agents on the dialysis of the iodide ion is shown in Fig. 1. Each curve represents the average of five determinations of the dialysis of the iodide ion. For control purposes the dialysis of the iodide ion exclusive of the influence of the suspending agent was determined. The data show that the release of the iodide ion is not linear with respect to time. All of the release patterns are asymptotic in character and approach the asymptote at approximately 80% release of the available iodide ion.

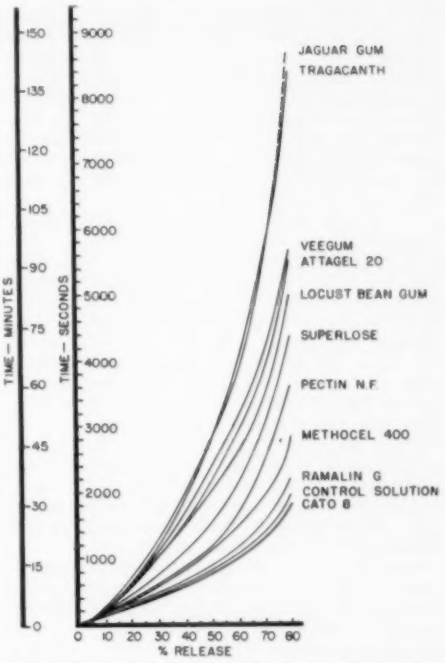

Fig. 1.—The influence of 1% concentrations of various suspending agents on the dialysis of the iodide ion.

If the period of time required to reach a given level of iodide dialysis from a suspending medium exceeded by 10% that which was required for the dialysis of an equal percentage of iodide ion from the control solution, the suspending agent was considered to have a significant influence on the release of the iodide ion from solution.

A test for homogeneity of variances among the means of the variable, time, was made at the 25% and also at the 75% release levels, and included all of the media and the control (11). In both instances it was established that the variances are heterogeneous. Therefore, the hypothesis that the interval of time required to obtain a given percentage of release of the iodide ion from the control solution and from a suspending medium are equal was tested using a t test (variances not equal) (12). The use of the t test was restricted to instances in which there was a reasonable doubt that a significant difference between the control and the media existed.

Only one of the media gave evidence of a capacity to increase the rate of passage of the iodide ion across the membrane. This agent, Cato 8, was shown by the application of a two-tailed t

[2] Knapp Monarch Co., St. Louis, Mo.
[3] Nuclear-Chicago Corp., Chicago, Ill.
[4] Esterline-Angus Co., Inc., Indianapolis, Ind.

test at an alpha level of 0.05 to effect a significant increase in the speed of the dialysis over that of the control. The reduction in the time required to release a specific amount of iodide ion was not significantly different from that of the control until 50% of the available iodide ion had dialyzed from the sample. The control time was approximately three minutes longer than that required to obtain the release of 80% of the available iodide ion from the media containing Cato 8.

The plot of the dialysis of the iodide ion from a 1% dispersion of Ramalin G shown in Fig. 1 indicates that the dialysis was retarded. The *t* test was used to test the means at the 25, 50, 75, and 80% release levels to determine if a significant difference exists between the means. A significant difference was found to exist at all levels tested. To obtain the dialysis of 80% of the iodide ion from the sample took about thirty-five minutes; for an equal dialysis from the control solution it took approximately thirty-three minutes.

All of the other media tested also retarded the dialysis of the iodide ion. Jaguar gum and tragacanth produced the greatest delay in the release followed by Veegum and Attagel 20, locust bean gum, Superlose, pectin N. F., Methocel 400, and Ramalin G in the order named.

The effect of concentration of the suspending agent on the dialysis rate of the iodide ion was also investigated. In Fig. 2 are shown the relative influences of 0, 0.5, 1.0, and 1.5% concentrations of Methocel 400 and Jaguar gum on the dialysis of the iodide ion. The data show that at the concentrations tested the agents retard the dialysis of the iodide ion, but that the dialysis rate is not proportional to the concentration of the agent in the media. Media containing 0.5 and 1.0% Methocel 400 released 80% of the contained iodide ion in approximately 2,850 seconds (forty-eight minutes), and that a 1.5% concentration of the agent took seven minutes longer. The control time was 1,977 seconds (thirty-three minutes).

Media containing 0.5% Jaguar gum released 80% of the available iodide ion in approximately 3,310 seconds (fifty-five minutes). The media containing 1.0 and 1.5% Jaguar gum showed no significant differences and released 80% of the iodide ion in 8,486 seconds (one hundred and forty minutes).

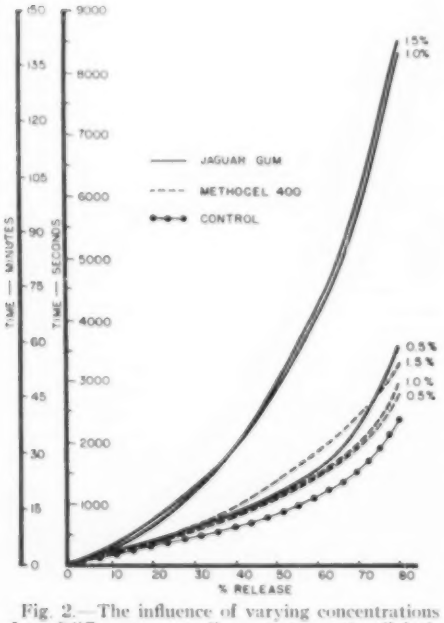

Fig. 2.—The influence of varying concentrations of two different suspending agents on the dialysis of the iodide ion.

CONCLUSIONS

1. The procedure used in this investigation has proved to be a satisfactory method for comparing the influence of suspending media on the dialysis of a soluble compound.

2. Different suspending agents were shown to affect the rate at which the iodide ion dialyzes from solution. The release rates indicate that the nature of the agent is a variable affecting the release of the compound.

3. Equal concentrations of the suspending agents tested affect the dialysis of the iodide ion in the following manner: Cato 8 increased the rate over that of the control; all others retarded the dialysis of the iodide ion. Jaguar gum and tragacanth produced the greatest delay followed by Veegum and Attagel 20; locust bean gum, Superlose, pectin N. F., Methocel 400, and Ramalin G, in the order named. Attagel 20 and Veegum affected iodide ion release to the same degree. Tragacanth U. S. P. and Jaguar gum also showed similar effects.

4. An increase in concentration of Methocel 400 and Jaguar gum does not alter the dialysis rate in proportion to the change in concentration.

5. The use of selected suspending agents enables the modification of release rates of certain soluble drugs from solution.

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Effect of Hydration on Hydrocholeresis in Rats*

By SISTER DANIEL JOSEPH, S. T. COKER, and L. L. EISENBRANDT

Hydration produces a significant increase in normal bile flow and a significant decrease in specific gravity. The hydrocholeretic response to sodium dehydrocholate is significantly greater in hydrated rats than in nonhydrated controls. Restimulation of nonhydrated animals does not increase bile flow above the peak of initial stimulation. Hydration does not appear to alter the mechanism of action of sodium dehydrocholate, since the onset and duration of action is the same in both control and hydrated animals. Only a potentiation of hydrocholeresis is evident.

MECHANISMS involved in bile formation have been studied by many investigators. Tanturi and Ivy (1) studied the effect of vascular changes in bile formation. Berman, *et al.* (2), noted that unconjugated, oxidized bile acids exert a greater osmotic effect, which results in excretion of a more hydrous bile than the conjugated acids. Cook, *et al.* (3), noted that dehydrocholic acid increased the excretory rate and biliary clearance of water, sodium, and chloride. They concluded that the mechanism of action of hydrocholeretic agents primarily involves stimulation of a filtration mechanism.

These observations as well as work in our laboratory led to the study of the effect of hydration on the hydrocholeretic response in rats.

EXPERIMENTAL

Twenty-five Sprague-Dawley albino rats weighing between 235–345 Gm. and maintained under standard laboratory conditions were divided into three groups. Anesthesia was initiated with ether and then replaced by thiopental sodium. The bile duct and trachea were cannulated. For the hydrated animals 0.1% thiopental sodium was dissolved in i. v. infusion fluid, and for nonhydrated animals 1% aqueous solution was given i. v. as needed. Hydration was effected by constant i. v. infusion of 2% of body weight with 0.9% sodium chloride over a period of one hour (4, 5). The infusion apparatus consisted of a Phipps and Bird syringe driver adapter with an electric kymograph. A 30-cc. syringe was used and the kymograph was operated at first speed; 0.079 cc. of fluid was administered per minute. Hydrocholeresis was produced by i. v. administration of 200 mg./Kg. of sodium dehydrocholate. Since it was observed that the maximal effect of sodium dehydrocholate was during the first thirty-minute period, all bile flow measurements were made at five minute intervals for thirty minutes after each treatment. Bile flow was reported in mg. wet weight/five-minute interval. Specific gravity of bile was determined by weighing in a tared syringe. Tests for significance were made using the Student t test and analysis of variance.

RESULTS AND DISCUSSION

A thirty-minute period of control or normal bile flow was determined in each of 25 animals prior to stimulation with sodium dehydrocholate. Group I consisted of 10 animals. After the control period (A–B, Fig. 1), they were subdivided into two groups; six which were stimulated only (B–D, Fig. 1) and four which were hydrated (F–G) and then stimulated (G–H). Group II consisted of 10 animals and differed from group I in that each animal was stimulated, allowed to return to normal, then hydrated for one hour and restimulated to obtain the hydrated-stimulated flow (A–H, Fig. 1). To determine whether the increase in hydrocholeresis after hydration and restimulation might be due to residual sodium dehydrocholate, a second dose of sodium dehydrocholate was administered to 5 nonhydrated animals in group III as soon as the original bile flow returned to normal (D–E, Fig. 1). No significant increase in bile flow was noted in these nonhydrated, restimulated animals. Summarized data on the effect of hydration on bile flow and specific gravity are in Table I.

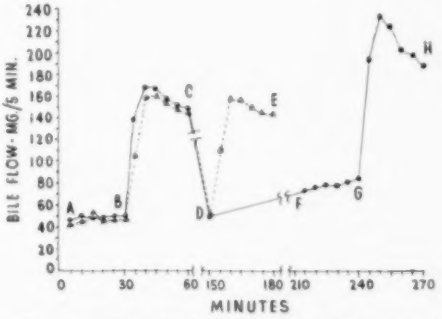

Fig. 1.—Mean Hydrocholeresis in 25 hydrated (·—·) and nonhydrated (△—△) rats. Flow areas A–B, normal; B–C, nonhydrated stimulated; C–D return to normal; D–E, nonhydrated restimulated; D–F, hydration priming; F–G hydrated; G–H, hydrated restimulated.

In every case there was a significant increase in bile flow and a decrease in specific gravity after one hour of hydration. Also, hydrocholeresis after hydration was significantly greater than in nonhydrated animals. The onset and duration of the hydrocholeretic response was the same after hydration, indicating that the effect produced by hydration is quantitative and not qualitative, with no change in the mechanism of action of the hydrocholeretic agent.

Sperber (6) considers that the primary event of bile formation might be the active transfer of bile

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TABLE I.—EFFECT OF HYDRATION ON HYDROCHOLERESIS IN RATS

	Mean Bile Flow, mg./30 min.	*P*[a] value	Increase from Normal, %	Mean Specific Gravity	*P* Value	Decrease from Normal, %
Group I[b] (10 rats)						
Normal	48.9 ± 4.8[c]			1.129		
Hydrated (4 rats)	80.5 ± 6.8	<0.01	65	1.118	<0.001	10
Stimulated (6 rats)	139.0 ± 12.5	<0.01	166	1.087	<0.001	37
Hydrated-stimulated	208.1 ± 11.8		326	1.067		55
Group II (10 rats)						
Normal	49.4 ± 2.6	<0.001		1.129	<0.001	
Stimulated	145.2 ± 15.6		218	1.082		42
Hydrated	80.2 ± 4.6	<0.05	62	1.106	<0.001	20
Hydrated-stimulated	212.9 ± 21.0		331	1.065		57
Group III (5 rats)						
Normal	46.7 ± 3.1			1.126		
Stimulated	145.2 ± 9.8	Not sig.	211	1.095	Not sig.	28
Nonhydrated-restimulation	146.5 ± 11.4		215	1.087		34

[a] Determined by analysis of variance, *t* test used for specific gravity.
[b] Group I, 10 rats, subdivided after normal period into four for hydration and hydrated stimulation; 6 for stimulation only.
[c] Standard error of mean.

acids from hepatic cells into bile capillaries. In the case of hydrated animals, a potentiation of hydrocholeresis could result from increased availability of water which readily passes into the bile capillaries in the presence of the osmotically-active sodium dehydrocholate. Brauer (7) feels that the mechanism of bile formation involves not only the active transport of bile acids, but also water, sodium, potassium, chloride, and glucose from blood to bile.

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A Gum from Rain Lily, *Cooperia pedunculata**

By WALLACE L. GUESS†, NATHAN A. HALL, and L. WAIT RISING

A heretofore undescribed plant principle was extracted from the bulbs of *Cooperia pedunculata;* the principle was purified and characterized as a new gum with several physical properties similar to those of other pharmaceutical gums. A preliminary chemical investigation of the gum from *C. pedunculata* (commonly known as rain lily) revealed that the gum gave reactions common to many of the known gums. It was also found that the gum was a polysaccharide and the principal sugar was identified as mannose by chromatographic procedures and the melting point of its phenylhydrazone. The infrared spectrum of a dried film of the gum was grossly similar to the spectra of most other gums commonly used in pharmacy, yet specific enough that a rapid identification of the gum would be possible. Rain lily gum exhibited rheological properties usually associated with natural hydrophilic colloids. The effect of aging, temperature, and pH on the viscosity of rain lily gum was also investigated.

GUMS are of major importance as pharmaceutical agents and they enjoy a large number of applications in other fields as well. For some gums, the United States is wholly dependent upon foreign sources; the annual importation of acacia and tragacanth has been reported as ten to fourteen million pounds (1, 2). The bulb of the rain lily, *Cooperia pedunculata*, which is native to the state of Texas, contains a highly viscous mucilaginous principle of potential pharmaceutical usefulness. The work reported here consists of the isolation and partial purification of this mucilaginous material, a potential domestic source of gum, and a preliminary investigation of its chemical nature.

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Abstracted in part from a thesis presented to the Graduate School, University of Washington by Wallace L. Guess in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
† Fellow of the American Foundation for Pharmaceutical Education. Present address: College of Pharmacy, University of Texas, Austin.
Presented to the Scientific Section, A. PH. A., Cincinnati Meeting, August 1959.

EXPERIMENTAL

Since the growing season of the rain lily plant is short and uncertain, large quantities of the bulbs were dug in the early Spring during the flowering and seed stages and stored in a freezing cabinet at −10°. The freshly dug bulbs were washed and the tops broken off, leaving a stem about 5–6 cm. long attached to the bulb. These bulbs were sealed in large plastic bags, frozen, and kept frozen until ready to be used. Little or no obvious decomposition of the bulbs occurred within a one-year storage period in the cold, however, it was found that if the bulbs were left at room temperature or rapidly dried in warm air, there was decomposition and a considerable loss of gum.

Isolation and Partial Purification of the Gum.—The outer coat was peeled from the frozen bulbs and 100-Gm. to 150-Gm. portions were chopped in a Waring Blendor with 500 ml. of purified water until homogeneous. Each portion was then diluted to approximately 750 ml. with water and placed in a stoppered Erlenmeyer flask. The flasks were placed in a refrigerator and allowed to stand for two days with occasional shaking. The liquid extract was removed from the pulp material by straining through gauze. The residue from each flask was again extracted by maceration with fresh water for two days and strained. The combined aqueous extracts were strained through muslin to remove the small amount of pulp which had passed through the gauze.

Microscopic examination of the aqueous extract showed the presence of starch granules and crystals of calcium oxalate. To remove the starch and most of the calcium oxalate, four 200-ml. centrifuge tubes were filled with the extract and centrifuged in an International centrifuge, Size 2, model V, at 2500 r. p. m. for fifteen minutes. This procedure was found to remove all of the starch granules (as determined by the iodine test) and most of the calcium oxalate, the remainder of which was removed during further purification.

The starch-free crude aqueous extract was poured into large, glass, flat-bottomed evaporating dishes (10 × 6 × 2 inches), frozen, and dried for approximately thirty hours in a Stokes freeze drying apparatus (model 2004LX3). The supporting trays for the dishes of frozen material were left at room temperature, while the lower, condensing trays were cooled by a refrigeration unit to −30° to −40°, and the pressure inside the chamber was reduced to approximately 1 mm. After about twelve hours the trays supporting the dishes of material were heated to 40° by circulating warm water and maintained at this temperature until the material completely dried. As the water evaporated from the dishes, the pressure inside the chamber dropped to a constant level of about 0.5 mm. The total time required for drying the extracts was about thirty hours. The product remaining in the dishes was a whitish-buff colored, light, flaky material that could be easily reduced to a fine powder by trituration. The average yield of dried, crude rain lily gum was about 10% of the total weight of the fresh frozen bulb weight.

Since the crude material was known to contain some calcium oxalate and possibly some soluble organic material, 20-Gm. portions of dried crude material were resuspended in 200 ml. of hot water, and this suspension was poured in a fine stream into one liter of warm 95% alcohol, with vigorous stirring. A white, ropy precipitate of mucilaginous material was collected, resuspended in hot water, and reprecipitated in warm alcohol. The precipitate was then washed with 95% alcohol and dried in the Stokes freeze drying apparatus as described previously. The average yield of purified rain lily gum from this procedure was about 25% of the total weight of the dried crude extract. Approximately three pounds of the purified rain lily gum was prepared, powdered in a ball mill, mixed thoroughly, and stored in a dark, cool place.

Chemical Examination of Rain Lily Gum.—One of the standard characteristics of acacia, tragacanth (3), and gums in general is precipitation from dispensions by lead subacetate. When 1 ml. of a 5% solution of lead subacetate was added to 5 ml. of a 4% dispersion of rain lily gum, heavy, white precipitate formed. To ascertain the carbohydrate nature of the material, a sample was tested for reducing sugars with Benedict's reagent. The reaction was negative, but on acidifying with hydrochloric acid, boiling the solution to hydrolyze the gum, and alkalinizing with sodium hydroxide, a positive Benedict's test was obtained. These tests indicated that part of the gum material under study was composed of a polysaccharide.

Partridge (4) was the first to report a separation of sugars by the use of paper chromatography. Since this worker's original report, numerous authors (5) have used paper chromatography to separate and identify simple sugars, sugar derivatives, and hydrolytic products of gums (6). To apply the paper chromatographic methods to rain lily gum, a hydrolysate was prepared by treating 1 Gm. of the gum with 1 *N* sulfuric acid (20 ml.) at 95° for ten hours. The excess sulfuric acid was neutralized with barium carbonate and the neutral solution filtered.

A sheet of Whatman No. 1 chromatography paper (29 cm. × 46 cm.) was prepared by drawing a pencil line 5 cm. from the bottom of the paper, and 0.1 ml. each of the hydrolysate and several sugar solutions were spotted along this line at 3-cm. intervals. The known sugar solutions (1 mg. in 1 ml. water) were galactose, mannose, rhamnose, and mixtures of these three sugars. The hexoses chosen as comparison sugars in this experiment were three sugars that are common to many of the known gums. Pentoses were not used as reference sugars since preliminary chromatographic work had shown that the R_f value of known samples was not near the R_f value for any sugar spots obtained from the rain lily hydrolysate.

The chromatographic procedure was carried out by the ascending method with two solvent systems: a mixture of *n*-butanol, glacial acetic acid, and distilled water (4:1:5) or ethyl acetate, distilled water, and pyridine (2:2:1). The bottom of a chromatographic jar was covered with the aqueous phase and the chamber sealed for twenty-four hours to ensure atmospheric saturation. One end of the prepared chromatography paper containing the sugar spots was then dipped in a Petri dish containing the mobile, nonaqueous phase of the above-mentioned solvents, the jar sealed, and the chromatograms allowed to develop for eighteen hours at 25°.

The paper was removed from the chromatographic jar and the solvent front marked. After being dried in the air, the paper was sprayed with 0.1 *N* aniline in glacial acetic acid and then heated in an oven at 100° for three minutes, resulting in distinct pink or brown spots from the reducing sugars (7). The distance the spots had moved from the starting line was measured and the R_f values calculated.

A comparison of the R_f values obtained (see Table I) with mannose and one of the sugars from the hydrolysate of rain lily gum showed that the values were nearly the same, whether the developing solvent was acidic or basic. These close values indicated that the hydrolysate of rain lily gum contained mannose. The second spot shown in the hydrolysate had an R_f value higher than any of the known sugars used, therefore no tentative identification of this spot was made. It was felt that the spot did not represent a uronic acid because R_f values reported (8) for several uronic acids were lower than the R_f values for the corresponding sugars while the R_f value for this unknown spot was higher than any of the test sugars.

Further evidence for mannose was provided by a phenylhydrazone derivative which showed an unchanged melting point when mixed with authentic mannose phenylhydrazone.

TABLE I.—A COMPARISON OF THE R_f VALUES OF CERTAIN KNOWN SUGARS WITH RAIN LILY HYDROLYSATE

Sugar	R_f Values: *n*-Butanol 40%, Glacial HAc, 10%, Water, 50%	R_f Values: Ethyl Acetate, 40%, Water, 40%, Pyridine, 20%
1. Galactose	0.203	0.582
2. Mannose	0.225	0.650
3. Rhamnose	0.367	0.736
4. Rain Lily	Spot a 0.223	Spot a 0.643
	Spot b 0.754	Spot b 0.915
Mixture of 1, 2, 3	1 0.199	1 0.579
	2 0.224	2 0.646
	3 0.363	3 0.721

Infrared Spectrum.—Newburger and co-workers (9, 10) have reported a method for identifying naturally-occurring gums by the use of infrared analysis. According to their method, a film of rain lily gum for examination was cast on a smooth spatula, coated with Desicote.[1] The spatula was dipped into a 4% dispersion of rain lily gum and dried over a steam bath. A piece of film large enough for analysis was removed with a razor blade, and this film was dried for six hours at 100°. The film was placed in a KBr cell which was positioned in a Baird-Atomic infrared spectrophotometer (model 4-55) and the infrared spectrum recorded.

Examination of the infrared spectrum (Fig. 1) reveals that its gross characteristics are very similar to that of acacia, tragacanth, guar, karaya, and many other gums; however, there are enough distinctive features in its spectrum that an identification of rain lily gum should be possible.

Viscosity Characteristics of Rain Lily Gum.—Although the reproducibility of rheological measurements made on dispersions of gums is generally poor due to numerous factors, it was felt that some useful information could be obtained by examining some of the flow characteristics of the rain lily gum dispersion. Measurements were made with a Brookfield viscometer, model LVF, with spindle No. 4, at 25° ± 1°. A 4% (w/v) dispersion of rain lily gum was prepared by placing the freeze-dried material in purified water, allowing it to hydrate in a refrigerator for forty-eight hours, and stirring with a mechanical stirrer. Dispersions of acacia (40%, w/v) and tragacanth (4%, w/v) were prepared similarly for comparison. In all measurements, after the samples had attained temperature equilibrium, the spindle was allowed to complete five revolutions before readings were taken and the values recorded represent the average of at least three readings. To assess the constancy of measurements, Brookfield readings at 30 r. p. m. were converted to "centipoises" by means of the Newtonian calibration chart supplied with the instrument. For several 4% rain lily gum dispersions prepared on different days, the average viscosity was 7,900 c. p. s. (standard deviation ±494).

Figure 2 illustrates the flow curves (up-curve only) of the rain lily gum dispersion compared to acacia and tragacanth. Rain lily gum dispersions show the typical pseudo-viscous or pseudo-plastic characteristics which are common to many plant gums. As expected, the viscosity of the acacia dispersion was too low for valid measurement within the experimental limitations.

Preliminary Pharmaceutical Evaluation.—Rain

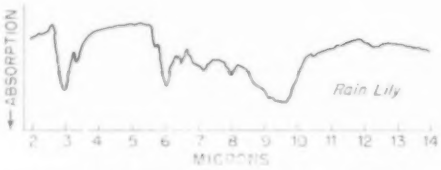

Fig. 1.—Infrared spectrum of rain lily gum.

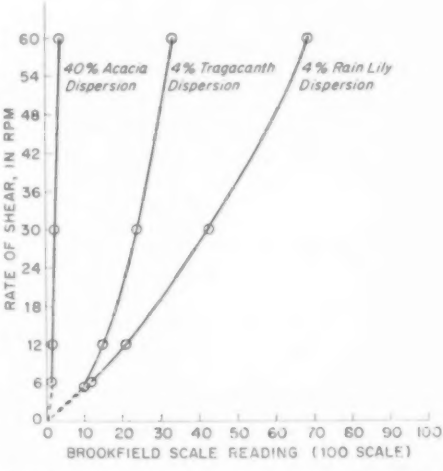
40% Acacia Dispersion
4% Tragacanth Dispersion
4% Rain Lily Dispersion
RATE OF SHEAR, IN RPM
0 6 12 18 24 30 36 42 48 54 60
0 10 20 30 40 50 60 70 80 90 100
BROOKFIELD SCALE READING (100 SCALE)

Fig. 2.—Flow curves for some gums. Brookfield viscometer, No. 4 spindle.

[1] Desicote is a silicone preparation purchased from Beckman Instruments, Inc., Pasadena, Calif.

lily gum was used to prepare 50% oil in water emulsions of liquid petrolatum and cod liver oil to examine its emulsifying ability. In this study rain lily gum (2.5%), tragacanth (2.5%), and acacia (25%) liquid emulsions were investigated over a two-month period by mean globule diameter method of Smith and Grinling (11). As a primary emulsifier, rain lily gum was comparable to tragacanth and inferior to acacia; thus it probably should be classified as an auxiliary emulsifier.

A number of suspension and semisolid cream formulas in which gums have been used were prepared with the rain lily gum. The preparations were generally satisfactory and further studies of the pharmaceutical applications of the gum are being made.

SUMMARY

A new gum from the bulbs of *Cooperia pedunculata* has been isolated and partially purified. A preliminary chemical investigation revealed that the gum was a polysaccharide containing mannose as a component monosaccharide. An infrared spectrum of the gum was similar to other gums and appeared to be characteristic enough for identification. The viscosity characteristics indicated that the gum was similar to tragacanth. A survey of possible pharmaceutical uses indicated that the gum could be classed as an auxiliary emulsifier and that further study of its pharmaceutical properties should be done.

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An Investigation of the Effect of Ultrasonic Waves on the Rates of Hydrolysis of Procaine and Butethamine Hydrochlorides*

By G. D. FENN and P. F. BELCASTRO

Data are presented showing the effect of ultrasonic waves on the rates of hydrolysis of procaine and butethamine[1] hydrochlorides. Under the experimental conditions employed, significant changes in the rates were not observed with the insonated samples.

A NUMBER of investigators (1–4) have reported that ultrasonic vibrations initiate or accelerate chemical reactions. Faust (5) investigated the chemical effects of ultrasound on glutathione and other compounds. Robert (6) discusses the oxidation of aromatic nuclei under the influence of ultrasonic vibrations and Prudhomme and Grabar (7) report depolymerization as a result of ultrasonic irradiation. Many such effects have been attributed to a local heating effect (2, 8, 9). This heating effect has been estimated to be as high as several hundred degrees and has been attributed to adiabatic compression of cavitation bubbles (10). In view of these investigations, the possibility of accelerating the rate of certain chemical reactions applied to pharmaceuticals was realized. This communication reports the effect of ultrasonic waves at a frequency of 400 kc. per second on the rates of hydrolysis of procaine and butethamine hydrochlorides, by comparison with noninsonated temperature accelerated hydrolysis. These comparative hydrolysis rates were investigated at several different hydrogen ion concentrations. It was hoped that the effects would be of sufficient magnitude to be of value in accelerated stability testing of pharmaceutical solutions.

* Received August 21, 1959 from the School of Pharmacy, Purdue University, West Lafayette, Ind.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
[1] Available under trade name Monocaine hydrochloride, Novocol Chemical Mfg. Co.

EXPERIMENTAL

Calibration of the Generator.—The ultrasonic generator employed in this study was constructed at Purdue University and was designed for use with a barium titanate transducer. The generator is rated at 250 watts with a variable frequency range. A Hypersonic transducer, model BU-301, with a focused bowl of barium titanate was employed. The generator was calibrated by determining the wattage produced at various rheostat settings by multiplying the power amplifier voltage by the power amplifier current, as shown by appropriate meters on the generator. To compensate for various trans-

mission losses, 65% of this calculated wattage was estimated to be the true energy reaching the sample (11). All insonation experiments were conducted at a frequency of 400 kc. per second and an energy level of approximately 125 watts at the focal point of the transducer.

Hydrolysis of Procaine Hydrochloride.—Twenty-five-milliliter samples of 0.1% procaine hydrochloride solution with various hydrogen ion concentrations were hydrolyzed under the influence of ultrasonic vibrations. The results obtained were compared with those for 100-ml. samples of 0.1% procaine hydrochloride samples hydrolyzed under the influence of temperature at 25.0° and at 30.0° ± 0.01°. Temperature control in the case of the noninsonated samples was maintained by use of a Sargent thermonitor controlled bath, model S-84810. The cooling coil of the transducer was not effective in maintaining precise temperature control of the insonated samples. The temperatures reported for the insonated samples represent the arithmetic mean of the temperatures observed at the time of sampling for analysis. The sample solutions were prepared by diluting a 1.0% solution of procaine hydrochloride to 0.1%, using an appropriate buffer solution to which 0.2% sodium bisulfite was added as an antioxidant. The pH of the resulting samples was determined before and after hydrolysis, using a Beckman model G pH meter. The composition of the buffers employed is shown by Table I.

TABLE I.—COMPOSITION OF MODIFIED SØRENSEN BUFFERS

Ingredient	Approximate pH				
	8	9	10	11	12
$NaHSO_3$, Gm./L.	2.00	2.00	2.00	2.00	2.00
H_3BO_3, Gm./L.	6.98	10.85	7.34	6.11	5.41
NaOH, Gm./L.	3.05	4.30	4.80	4.80	4.80
0.1 *M* HCl, ml./L.	437.50	125.00	0.00	0.00	0.00

The analytical procedure for the determination of residual procaine hydrochloride was essentially that of Higuchi (12), modified as follows: A 1-ml. portion of the sample was diluted to 100 ml. with a pH 9.5 buffer composed of 96.131 Gm./L. of H_3BO_3 and 4.000 Gm./L. of NaOH. The absorbance of the resulting solution was then determined at wavelengths of 287 mμ and 271.5 mμ using a Beckman model DU spectrophotometer. The per cent residual ester was then calculated according to the following formula as reported by Higuchi (12):

$$\% \text{ residual ester} = 100 \times \frac{(k - k_{PABA})}{(k_{Proc} - k_{PABA})}$$

where k = the observed absorbance of the partially hydrolyzed sample diluted to 0.001% with respect to original procaine hydrochloride; k_{PABA} = the absorbance of *p*-aminobenzoate ion equivalent to 0.001% concentration of procaine hydrochloride; and k_{Proc} = the absorbance of 0.001% procaine hydrochloride, all readings being taken at a fixed wavelength and pH 9.5.

The absorbances determined at 271.5 mμ served as an internal check on any side reactions and limited the useful data taking time since the molar extinction coefficients for procaine hydrochloride and PABA are the same at this wavelength. Figure 1 shows the first-order hydrolysis observed at pH 9.9 under the various conditions employed.

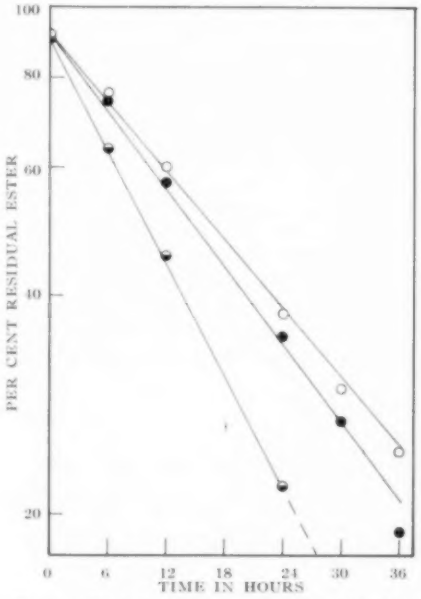

Fig. 1.—Hydrolysis of procaine hydrochloride in solution at pH 9.9; ○, noninsonated at 25.0°; ◒, noninsonated at 30.0°; ●, insonated at 26.7°.

Hydrolysis of Butethamine Hydrochloride.—The procedure followed for butethamine hydrochloride was similar to that used for procaine hydrochloride. No solutions of butethamine hydrochloride were hydrolyzed at pH 11 or 12 since preliminary experimentation indicated that the half lives would be less than fifteen minutes. The formula for calculation of per cent residual procaine ester was modified as follows for use with butethamine hydrochloride.

$$\% \text{ residual ester} = 100 \times \frac{(k - k_{PABA})}{(k_{Bute} - k_{PABA})}$$

where k = the observed absorbance of the partially hydrolyzed sample diluted to 0.001% with respect to original butethamine hydrochloride; k_{PABA} = the absorbance of *p*-aminobenzoate ion equivalent to 0.001% concentration of butethamine hydrochloride; and k_{Bute} = the absorbance of 0.001% butethamine hydrochloride, all readings being taken at a fixed wavelength and pH of 9.5.

It was determined that the wavelength of maximum absorption for butethamine hydrochloride is 287 mμ and the isoabsorptive point with PABA occurs at 266 mμ. Figure 2 shows the first-order hydrolysis observed at pH 7.9 under the various conditions employed.

Comparison of Half Lives.—In order to show the comparative rates of hydrolysis of the various samples under all the experimental conditions employed

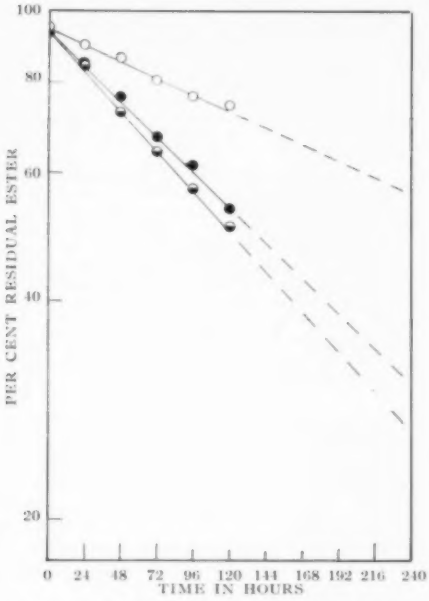

Fig. 2.—Hydrolysis of butethamine hydrochloride in solution at pH 7.9; ○, noninsonated at 25.0°; ◒, noninsonated at 30.0°; ●, insonated at 29.8°.

the half lives of all samples were calculated using the following formula (13):

$$t_{1/2} = \frac{\log 2}{-m}$$

where $t_{1/2}$ = the half life and m = the slope of the line obtained by plotting time against the log of the per cent residual ester. The value for m was calculated from the following formula (14):

$$m = \frac{N\Sigma xy - \Sigma x\Sigma y}{N\Sigma x^2 - (\Sigma x)^2}$$

where N = the number of observations, x = the numerical value of the point on the x axis, and y = the numerical value of the point on the y axis. Tables II and III show the comparative half lives for all samples under the various conditions employed in this study.

DISCUSSION

These data would indicate that the only effect of ultrasonic vibrations on the systems employed in this study is probably due to the overall increase in the temperature of the solution and that the local heating effect is not of sufficient magnitude to produce an abnormal rate of degradation. This can be explained in a number of ways. First, the local thermal energy developed would be at the focal point of the ultrasound which has a cross-sectional area of 0.5 cm.^{2} Second, due to the agitation of the solution as a result of cavitation, this heat is no doubt rapidly dispersed throughout the system, accounting for the overall rise in the temperature of the solution. Third, since the systems employed were fluid in nature, less heat would be generated as a result of intermolecular friction than would be true for solid or semisolid systems.

TABLE II.—HALF LIVES OF SAMPLES OF PROCAINE HYDROCHLORIDE AT VARIOUS pH'S

pH	$t_{1/2}$ in Hours		
	25.0°C.	30.0°C.	Insonated, °C.[a]
8	249.09	114.02	106.95, 31.9
9	35.62	20.74	30.25, 25.9
10	18.01	11.28	15.81, 26.7
11	12.15	7.03	10.92, 25.8
12	1.69	1.10	1.67, 25.3

[a] Arithmetic mean of temperatures observed at time of sampling for analysis, a minimum of 6 observations.

TABLE III.— HALF LIVES OF SAMPLES OF BUTETHAMINE HYDROCHLORIDE AT VARIOUS pH'S

pH	$t_{1/2}$ in Hours		
	25.0°C.	30.0°C.	Insonated, °C.[a]
8	329.03	132.65	152.94, 29.8
9	22.63	11.25	18.69, 25.8
10	2.54	1.48	1.85, 27.3

[a] Arithmetic mean of temperatures observed at time of sampling for analysis, a minimum of 6 observations.

SUMMARY

1. The purpose of this study was to determine the effect of ultrasonic waves on the rates of hydrolysis of certain pharmaceuticals in aqueous solution. The rates of insonated samples were compared to the rates of noninsonated samples under similar conditions. Ultrasonic oxidation of the compounds was prevented by the addition of an antioxidant.

2. No significant differences were noted between the rates of hydrolysis of insonated and noninsonated samples other than those attributable to slight increases in the overall temperatures of the insonated solutions.

3. Under the experimental conditions described in this paper, it does not appear that ultrasonic waves have any significant influence on the rates of hydrolysis of procaine or butethamine hydrochlorides.

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A Preliminary Study of the Alkaloidal Principles of *Ceanothus Americanus* and *Ceanothus velutinus**

By CHARLES W. ROSCOE† and NATHAN A. HALL

The presence of a complex mixture of alkaloids in the root barks of *Ceanothus Americanus* and *Ceanothus velutinus* has been demonstrated by means of circular paper partition chromatography. Preliminary fractionation studies have yielded one crystalline alkaloid, m. p. 251–252° (K), from *Ceanothus velutinus*. The alkaloid was partially characterized by its infrared spectrum. The total alkaloid fraction from *Ceanothus Americanus* and the ether-soluble and ether-insoluble alkaloid fractions from *Ceanothus velutinus* were tested for hypotensive activity and found to exhibit insignificant activity at the reported dosage levels. Since previous investigators have reported on the hypotensive activity shown by various alkaloidal extracts from *Ceanothus Americanus* it is recommended that the fractionation studies be continued in the hope of isolating sufficient quantities of individual alkaloids for further pharmacological testing and chemical characterization.

THE RESULTS of early chemical investigations of *Ceanothus Americanus* (1–4) suggested the presence of a complex mixture of alkaloids in the root bark of this shrub. The only reported attempt to resolve the alkaloid mixture by chromatographic methods was unsuccessful (5). In the light of these results it seemed that a reexamination of the alkaloidal material should be undertaken.

Other species of *Ceanothus* have received little or no attention from investigators. The species *Ceanothus velutinus* grows so prolifically in easily accessible areas in the state of Washington as to warrant more than casual interest. Richards and Lynn (6), in 1934, submitted evidence for the presence of alkaloidal material in this species but no subsequent investigations have been reported.

The primary objective of this study was that of attempting to demonstrate the homogeneous or heterogeneous nature of the alkaloidal principles in the two plant species by means of improved chromatographic techniques. It was also hoped that some pure alkaloids might be isolated for use in future chemical characterization and biological activity studies. Previous reports on the hypotensive activity shown by alkaloidal extracts obtained from *Ceanothus Americanus* (5, 7, 8) served to lend additional impetus to the study.

EXPERIMENTAL

Source of Plant Material

The roots of *Ceanothus velutinus* were collected in the immediate vicinity of U. S. Highway 2, approximately 15 miles east of the summit of Stevens Pass in the state of Washington. The bark was removed from the freshly dug roots and allowed to dry in air prior to comminution in a Wiley mill fitted with the smallest size screen. The material was collected at various times during the summer months. No attempt was made to ascertain the optimum harvest time. Herbarium samples of *Ceanothus velutinus* were authenticated by Dr. C. L. Hitchcock, Department of Botany, University of Washington.

The root bark from *Ceanothus Americanus* was supplied by Flint, Eaton & Co., Decatur, Ill.

Isolation of Alkaloid Fractions

Preliminary studies indicated that the initial extraction of the alkaloids from either plant species was best accomplished with methanol or ethanol. No prior alkalinization was necessary. Defatting of the drug was found to be desirable since appreciable quantities of vegetable fat or wax were present.

The general scheme for obtaining the alkaloid fractions is outlined in Fig. 1. Amorphous alkaloid fractions were isolated from the root bark of *C. velutinus* and *C. Americanus* in yields of 0.20% and 0.16%, respectively. Specific rotations were found to be as follows: *C. velutinus*, $[\alpha]_D^{25} -205°$ ($CHCl_3$); *C. Americanus*, $[\alpha]_D^{25} -150°$ ($CHCl_3$).

The acid fractions obtained from the ether extracts (Fig. 1) are currently under investigation.

Resolution of Alkaloid Mixtures by Circular Paper Partition Chromatography

Method.—Manian (5) screened an extensive number of solvent systems in an unsuccessful attempt to resolve the alkaloidal mixture in *C. Americanus* by means of paper-strip chromatography. Likewise, we investigated a number of solvent systems and techniques without success. Finally, some degree of resolution was achieved with the ascending, moist, buffered paper technique which Levine and Fischbach (9) employed for the resolution of a mixture of veratrum alkaloids with *n*-butyl acetate-*n*-butanol solvent systems containing acetic or formic acid. However, the resulting chromatograms exhibited elongated, diffuse spots with considerable tailing which made it difficult to locate individual spots with any degree of certainty. By utilizing the basic features of this method and incorporating modifications of the circular paper

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This paper is abstracted in part from a dissertation presented to the Graduate School of the University of Washington by Charles W. Roscoe in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

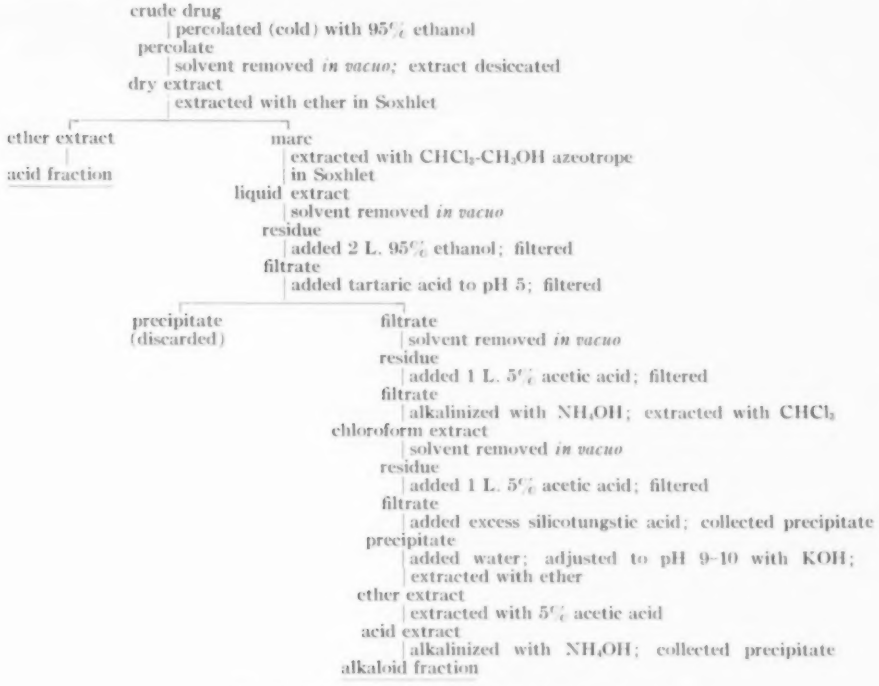

Fig. 1.—Scheme for isolation of alkaloid fractions.

technique described by Rao (10), satisfactory resolution was eventually achieved. Optimum results were obtained using the resolving solvent system *n*-butyl acetate : *n*-butanol : acetic acid, 25:5:1 and 25:1:1 (by volume).

Apparatus and Materials.—The chromatography container used in these studies consisted simply of a glass casserole cover about 18 cm. in diameter, which served as the reservoir for the developing solvent, and a heavy desiccator lid which served as the cover. The edges of the two parts were ground so that a tight seal resulted when they were fitted together.

The solvents, *n*-butyl acetate and *n*-butanol, of the mobile phase were purified by distillation through a Todd precise fractionation assembly. Reagent grade glacial acetic acid was used without further treatment. McIlvaine's phosphate-citrate buffer (11), pH 3.5, was used as the stationary phase.

Procedure.—A circle 18 cm. in diameter was drawn on a sheet of Whatman No. 1 filter paper. A wick was fashioned by cutting a flap 1 cm. wide and 2 cm. long from the edge of the circle toward its center. The flap was then folded downward, perpendicular to the sheet, and a strip of filter paper stapled to the lower end of the flap in order for the wick to extend into the developing solvent. An arc of 2 cm. radius with the fold of the wick as its center was drawn toward the center of the paper. Solutions of the alkaloid mixtures were applied at two points, 1.4 cm. apart, along the arc. With development of the chromatograms the components of the mixtures appeared to progress radially along imaginary lines intersecting the starting points and the center of the arc. The most consistent R_f values were obtained by measuring distances along these lines.

The paper, prepared in the described manner, was quickly drawn through the aqueous phosphate-citrate buffer solution and the excess moisture removed by blotting between paper towels. Quantities of 200 to 400 mcg. of the alkaloid mixtures in chloroformic solution were spotted on the moist paper along the pencil-drawn arc. The paper was then exposed to the air for several more minutes and when it had attained the desired degree of wetness, as determined largely by the feel of the paper, it was sandwiched between the casserole cover and the desiccator lid with the wick dipping into the developing solvent. The solvent front was allowed to travel about 14 cm., after which the chromatogram was removed, dried, and sprayed with a 0.01% chloroformic solution of bromophenol blue. On humidifying the chromatogram with a flow of steam the alkaloids appeared as blue bands on a yellow background. A reproduction of a typical chromatogram produced in this manner is shown in Fig. 2.

Results.—Using the resolving solvent system *n*-butyl acetate : *n*-butanol : acetic acid, 25:5:1, five bands were observed on chromatograms of the *C. Americanus* mixture and six bands on chromatograms of the *C. velutinus* mixture. The results are summarized in Table I.

Using the resolving solvent system *n*-butyl acetate : *n*-butanol : acetic acid, 25:1:1, seven bands

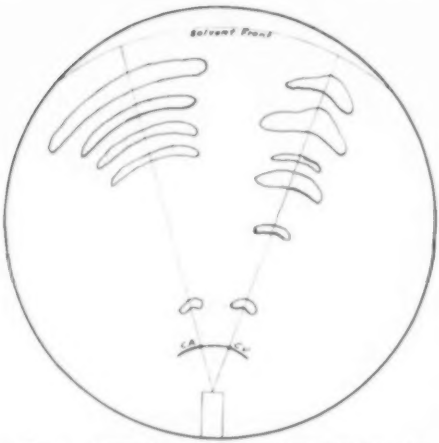

Fig. 2.—Reproduction of a typical alkaloid chromatogram developed with the solvent system *n*-butyl acetate : *n*-butanol : acetic acid (25:5:1, by volume). CA, *Ceanothus Americanus;* CV, *Ceanothus velutinus.*

TABLE I.—R_f VALUES CALCULATED FROM CHROMATOGRAMS DEVELOPED WITH THE SOLVENT SYSTEM *n*-BUTYL ACETATE : *n*-BUTANOL : ACETIC ACID, 25:5:1 (BY VOLUME)

Band No.	*Ceanothus Americanus* Mean Rf[a]	*Ceanothus Americanus* Av. Error, ±%	*Ceanothus velutinus* Mean Rf[a]	*Ceanothus velutinus* Av. Error ±%
1	0.17	4.7	0.18	5.6
2	0.64	3.1	0.42	1.4
3	0.72	2.8	0.60	0.7
4	0.81	2.5	0.66	1.5
5	0.92	1.1	0.80	1.0
6	...	..	0.92	2.2

[a] Average of five determinations.

appeared on chromatograms of the *C. americanus* mixture and five bands on chromatograms of the *C. velutinus* mixture. The results are summarized in Table II.

Fractional Solution of Alkaloid Mixtures with Ether

In view of the apparent complexity of the alkaloid mixtures in both plant species it was hoped that a preliminary crude separation might serve to facilitate further fractionation by more elaborate methods.

Method.—The mixture of alkaloids from *C. Americanus* was subjected to continuous extraction in a Soxhlet apparatus with anhydrous, peroxide-free ether for a total of five days. The extractive obtained after a forty-eight-hour extraction period was designated fraction 1; the extractive obtained after an additional seventy-two-hour extraction period was designated fraction 2. The remaining residue was designated fraction 3.

The mixture of alkaloids from *C. velutinus* was separated into two ether fractions by a similar procedure. The ether extractive obtained after a 96-hour extraction period was designated fraction 1; the remaining residue, fraction 2.

TABLE II.—R_f VALUES CALCULATED FROM CHROMATOGRAMS DEVELOPED WITH THE SOLVENT SYSTEM *n*-BUTYL ACETATE : *n*-BUTANOL : ACETIC ACID, 25:1:1 (BY VOLUME)

Band No.	*Ceanothus Americanus* Mean Rf[a]	*Ceanothus Americanus* Av. Error, ±%	*Ceanothus velutinus* Mean Rf[a]	*Ceanothus velutinus* Av. Error, ±%
1	0.18[b]	9.4	0.08	5.3
2	0.24[b]	4.2	0.41	2.4
3	0.40	3.0	0.60	1.7
4	0.54	1.9	0.68	1.2
5	0.67	1.5	0.79	0.4
6	0.81	0.7	..	..
7	0.93	0.6	..	..

[a] Average of five determinations.
[b] Detected under ultraviolet light.

Results.—Fractions 1, 2, and 3 from *C. Americanus* represented approximately 61, 22, and 17%, respectively, of the total weight of the original mixture. Specific rotations taken in chloroform at 26 ± 2° were found to be −178, −167, and −100° for fractions 1, 2, and 3, respectively. Paper chromatograms of fraction 1, developed with the solvent system *n*-butyl acetate : *n*-butanol : acetic acid, 25:1:1, showed the presence of seven bands corresponding closely to those listed in Table II. An eighth band (R_f 0.09), which had not been previously detected on chromatograms of the original mixture, was also found. Four bands appeared on chromatograms of fraction 2 and two bands on chromatograms of fraction 3.

Fractions 1 and 2 from *C. velutinus* represented approximately 44 and 56%, respectively, of the weight of the original mixture. Specific rotations taken in chloroform at 26 ± 2° were found to be −215 and −225° for fractions 1 and 2, respectively. With the above solvent system paper chromatograms of fraction 1 showed the presence of five bands corresponding closely to those listed in Table II. Two additional bands (R_f's 0.14 and 0.24) were detected by examining the chromatograms under ultraviolet light. Three (and possibly four) bands could be detected on chromatograms of fraction 2.

Preliminary Fractionation by Column Adsorption Chromatography

Method.—The general procedure outlined by Reichstein and Shoppee (12) for the fractional elution of steroids from columns of alumina with an eluotropic series of solvents was similarly employed in an attempt to effect a further separation of the alkaloids in each of the ether fractions. Woelm aluminum oxide[1] (nonalkaline, activity grade 1) was used as the adsorbent in these studies. The fractions obtained by this method were examined individually by means of their paper chromatograms and specific rotations. The initial results of these studies are briefly reported here. A more detailed report will be published at a later date when further studies have been completed.

Results.—The specific rotations of the *C. Americanus* fractions progressively decreased from −222 to −47° in the order that they were eluted from the alumina column. One of the amorphous fractions, which appeared to contain a single component on the basis of its paper chromatogram, melted at

[1] Available from Research Specialties Co., 2005 Hopkins Street, Berkeley 7, Calif.

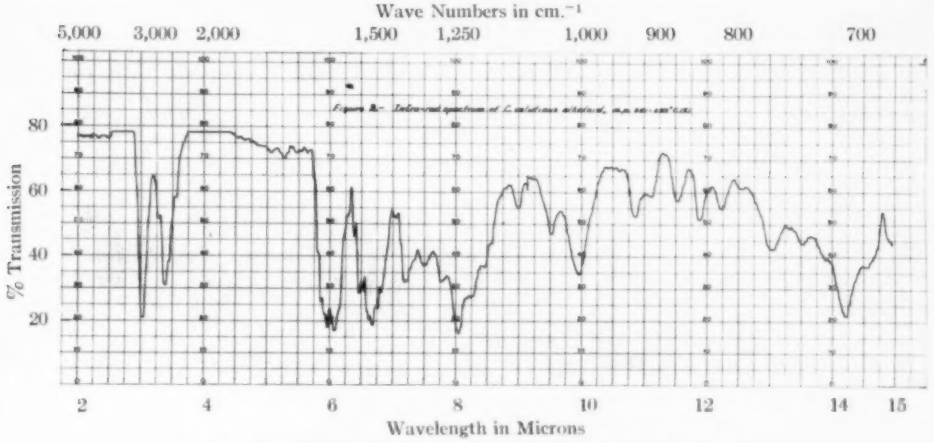

Fig. 3.—Infrared spectrum of *C. velutinus* alkaloid, m. p. 251–252° (K).

138–139° (K) but attempts to crystallize it were unsuccessful. All other fractions contained two or more major components.

The specific rotations of the *C. velutinus* fractions ranged from −292 to −45°. One of the crystalline fractions appeared to consist of one major component. The colorless, acicular crystals melted at 251–252° (K) after three recrystallizations from absolute ethanol. All other fractions contained two or more major components.

Infrared Spectrum of C. velutinus Alkaloid.—The infrared spectrum of the crystalline *C. velutinus* alkaloid, m. p. 251–252° (K), is shown in Fig. 3. The spectrum was taken on a solid sample in the form of a potassium bromide tablet using a Perkin-Elmer infrared spectrophotometer, model 21, equipped with a sodium chloride prism.

The single strong band at 3,300 cm.$^{-1}$ falls within a frequency range which is usually assigned to the N—H stretching vibrations of secondary amines, secondary amides, and imines, although hydrogen-bonded OH groups may also absorb at this frequency. The band near 2,900 cm.$^{-1}$ occurs at a frequency normally expected for C—H stretching vibrations.

The intense absorption at 1,680 cm.$^{-1}$ suggests the presence of a conjugated carbonyl group. The equally intense absorption at 1,640 cm.$^{-1}$ might be considered indicative of the carbonyl absorption of an amide group since all amides examined in the solid state have been found to absorb strongly near this frequency (13). The alternative possibility that the 1,640 cm.$^{-1}$ band could arise from the C=C stretching vibrations of an $\alpha\beta$ unsaturated keto group should also be considered. The weaker absorption near 1,550 cm.$^{-1}$ might also be associated with an amide group (14).

Some indication for the presence of an aromatic structure is found in the absorption observed in the 1,600–1,500 cm.$^{-1}$ region in conjunction with the sharp, relatively weak band which appears as a shoulder on the high frequency side of the 2,900 cm.$^{-1}$ band (15). The band in the vicinity of 1,390–1,380 cm.$^{-1}$ occurs in a region which has often been assigned to the C—CH_3 group.

The intense absorption at 1,240 cm.$^{-1}$ falls within a frequency range normally assigned to the C—O stretching vibrations of aryl and aralkyl ethers as well as phenols, esters, acids, and other compounds possessing the =C—O structure (16). The absorption near 1,110 cm.$^{-1}$ could be due to an ether group.

The absorption bands near 870 and 740 cm.$^{-1}$ in conjunction with those in the 1,600–1,500 cm.$^{-1}$ region give some reason to suspect the presence of an aromatic heterocyclic ring system. The strong band near 705 cm.$^{-1}$ could be due to out-of-plane =C—H bending vibrations.

PHARMACOLOGY

Although the literature contains several reports attesting to the hypotensive activity shown by various alkaloidal extracts derived from *C. Americanus*, no report on the pharmacological testing of *C. velutinus* extracts has been found. The results of a preliminary testing[1] of the ether-soluble and ether-insoluble alkaloid fractions of *C. velutinus* for hypotensive activity are reported here. A sample of the total alkaloids of *C. Americanus* was also tested. All tests were conducted on the anesthetized dog.

Total Alkaloids of C. Americanus and Ether-Insoluble Alkaloids of C. velutinus.—Intravenous doses below 5 mg./Kg. produced no significant change in blood pressure. At dose levels of 5 and 10 mg./Kg. a slight fall in blood pressure (15–20 mm. Hg) of brief duration (one to two minutes) was observed.

When administered intraduodenally at a dose level of 10 mg./Kg. the ether-insoluble alkaloids of *C. velutinus* produced no change in blood pressure during a period of eighty-five minutes. The total alkaloids of *C. Americanus* caused a small increase in cardiac rate whereas the ether-insoluble fraction from *C. velutinus* produced no significant change. Neither of these substances interfered with adrenaline pressor responses nor did they show significant ganglionic blocking action.

Ether-Soluble Alkaloids of C. velutinus.—Intravenous doses of 1, 5, and 10 mg./Kg. evolved a fall in blood pressure (10–45 mm. Hg) of brief duration (one to eight minutes) accompanied by a

[1] The authors are indebted to the pharmacology staff of Mead Johnson & Co. for the pharmacological testing of the alkaloid samples for hypotensive activity.

small decrease in cardiac rate. This response resembles that observed after the i. v. administration of many types of drugs and was not considered to be significant. This fraction did not interfere with adrenaline pressor responses and exhibited no significant ganglionic blocking action.

DISCUSSION

The results of the fractional solution with ether indicated that the majority of the alkaloids in each plant species resided in the more ether-soluble fractions. The appearance of previously undetected bands on paper chromatograms of the ether fractions suggested that there may be at least eight alkaloids in the root bark of *C. Americanus* and seven alkaloids in the root bark of *C. velutinus*, although the possibility that some of the bands might represent basic artifacts was considered.

The initial results of the fractionation by column chromatography indicated that this procedure might be employed, with certain refinements, for the final resolution of the alkaloid mixtures on a preparative scale. Unfortunately, the crystalline *C. velutinus* alkaloid, m. p. 251–252° (K), was isolated in extremely small yield and additional characterization studies were not possible due to the lack of sufficient material.

The insignificant hypotensive activity shown by the sample of total alkaloids from *C. Americanus* is not in agreement with the findings of previous investigators. However, this may be due to the administration of lower dosages. Further testing at higher dosage levels was prevented by the lack of sufficient material. The possibility that the alkaloids might possess activity of a cumulative nature was considered.

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The Synthesis and Antifungal Evaluation of Certain Acetylenic Compounds*

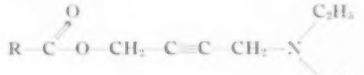
By JAMES A. WATERS† and GAIL A. WIESE

A number of 4-diethylamino-2-butynyl esters and 1,4-bis-N-substituted amino-2-butynes were prepared. The compounds, in the form of their hydrochloride and/or methiodide salts, were tested *in vitro* against three fungi causing dermatomycoses. Several compounds exhibited good activity in comparison to the control compound, sodium undecylenate. The most effective compound in this investigation was 4-diethylamino-2-butynyl *o*-toluate hydrochloride.

IN PAST YEARS, relatively few investigators have studied the antifungal activity of compounds containing an acetylenic component. The most recent paper in which a number of acetylenic compounds were evaluated was by Tanaka, *et al.* (1). The compounds were patterned after an acetylenic compound the same laboratory had previously isolated from the essential oil of *Artemisia capillaris*, which was reported to possess a high degree of antifungal activity (2).

It was of particular interest to note that in certain instances where acetylene carboxylic acids were studied for antifungal activity (3, 4), the free acids exhibited little fungistatic activity but when esterified they were highly fungistatic.

In this investigation a number of acetylenic aminoesters were prepared, of the general formula shown below.



R = alkyl, alkenyl, or aromatic

Esters of the same pattern were of considerable interest to Marszak, *et al.* (5), and Biel, *et al.* (6), as potential pharmacodynamic agents. We were interested in studying the structure—antifungal activity relationships afforded by these three general types of esters.

A few acetylenic diamines were also prepared. The free bases were converted into their hydrochloride and/or methiodide salts. The compounds prepared in this investigation are summarized in Tables I and II.

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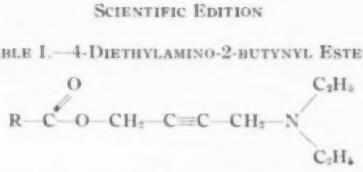
Abstracted from a dissertation submitted to the Graduate College of the State University of Iowa by James A. Waters in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August 1959.
† Fellow of the American Foundation for Pharmaceutical Education, 1958–1959. Present address: Department of Chemistry, University of Michigan, Ann Arbor.

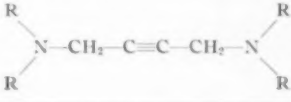
TABLE I.—4-DIETHYLAMINO-2-BUTYNYL ESTERS



Ester	Yield, %	B.p.,[a] °C.	Salt	No.	Yield, %	M.p.,[b] °C.	Formula	Analysis of N, %: Calcd.	Found
Acetate[n]	63.0	85–89[m]	HCl	1	54.6	93–94	$C_{10}H_{10}NO_2Cl$[b]	6.38	6.25
Propionate	72.0	78–80	...	...	...	...	...	...	...
n-Butyrate	75.4	78–81.5	...	...	...	...	...	...	...
n-Hexanoate	81.0	94–95	HCl	2	76.2	62–63	$C_{14}H_{26}NO_2Cl$[c,h]	5.08	5.18
n-Octanoate	89.9	115–117	CH_3I	3	37.2	57–58.5	$C_{17}H_{32}NO_2I$[d]	3.42	3.37
Crotonate	47.8	98–104	HCl	4	62.6	80–83	$C_{12}H_{20}NO_2Cl$[b]	5.70	5.54
10-Undecenoate	70.9	[f]	CH_3I	5	63.0	75–77	$C_{20}H_{36}NO_2I$[c]	3.12	3.01
Sorbate	74.6	115–120	HCl	6	53.5	97–98	$C_{14}H_{22}NO_2Cl$[b]	5.15	5.19
			CH_3I	7	32.2	100–101	$C_{15}H_{24}NO_2I$[c]	3.71	3.73
Benzoate[o]	65.3	125–126	HCl	8	75.0	98	$C_{15}H_{20}NO_2Cl$[b]	4.96	4.80
			CH_3I	9	44.4	91–92	$C_{16}H_{22}NO_2I$[c]	3.62	3.78[i]
o-Toluate	73.6	121–121.5	HCl	10	82.4	135–136	$C_{16}H_{22}NO_2Cl$[b]	4.74	4.54
			CH_3I	11	90.6	106–108	$C_{17}H_{24}NO_2I$[c]	3.50	3.42
m-Toluate	81.8	124–126	HCl	12	27.6	160–162	$C_{16}H_{22}NO_2Cl$[b,k,l]	4.74	4.91
			CH_3I	13	77.7	116–118.5	$C_{17}H_{24}NO_2I$[c]	3.50	3.40
p-Toluate	64.3	133–134	HCl	14	93.0	113–114.5	$C_{16}H_{22}NO_2Cl$[b]	4.74	4.65
p-Nitrobenzoate	61.7	46–47[g]	HCl	15	56.3	136–137	$C_{15}H_{19}N_2O_4Cl$[b]	8.57	8.25
			CH_3I	16	66.6	134–136	$C_{16}H_{21}N_2O_4I$[e]	6.48	6.32

[a] Boiling points at 0.3–0.5 mm. pressure, unless otherwise indicated. [b] Recrystallized from an absolute ethanol-anhydrous ether mixture. [c] From absolute ethanol. [d] From absolute methanol-anhydrous ether mixture. [e] No solvent was found satisfactory to recrystallize this compound. [f] A viscous oil which could not be distilled at 0.3–0.5 mm. without decomposition. [g] Melting point. [h] Extremely hygroscopic. [i] Nitrogen analyses by a semimicro Kjeldahl method, unless otherwise indicated. [j] Analysis performed by Galbraith Laboratories, Knoxville, Tenn. [k] Melting points are uncorrected and were taken in capillary melting point tubes. [l] Melting point taken in a closed capillary tube. [m] B. p. at 1 mm. pressure. [n] The free aminoester reported by Marszak, I., *et al.*, *Compt. rend.*, **226**, 1289(1948); b. p. 94° (0.5 mm.). [o] *Ibid.*; reported b. p. 163° (0.6 mm.).

TABLE II.—1,4-BIS-N-SUBSTITUTED AMINO-2-BUTYNES



	Yield, %	B. p.,[a] °C.	Salt	No.	Yield, %	M. p.,[b] °C.	Formula	Analysis of N, %: Calcd.	Found
Diethylamino[c]	75.0	73–79	Di-HCl	17	63.5	204–205	$C_{12}H_{26}N_2Cl_2$[c]	10.40	10.06
			Di-CH_3I	18	91.3	213.5–214	$C_{14}H_{30}N_2I_2$[f]	5.83	5.51
Pyrrolidino[d]	92.5	98–102	Di-HCl	19	74.8	222	$C_{12}H_{22}N_2Cl_2$[g]	10.56	10.49[i]
			Di-CH_3I	20	80.5	246	$C_{14}H_{26}N_2I_2$[g,h]	5.88	5.85[i]

[a] Boiling points at 0.3–0.5 mm. pressure. [b] All melting points with decomposition. [c] Reported b. p. 110° (10 mm.); Wille, F., *et al.*, *Ann.*, **591**, 177(1955). [d] Reported b. p. 93–95° (0.1 mm.); Biel, J., and DiPierro, F., *J. Am. Chem. Soc.*, **80**, 4609(1958). [e] Recrystallized from anhydrous ether–methanol mixture. [f] From absolute ethanol. [g] From absolute methanol. [h] Reported m. p. 239–240°; ref., see footnote d. [i] Analyzed by Galbraith Laboratories, Knoxville, Tenn.

ANTIFUNGAL EVALUATION

The compounds were tested *in vitro* against *Trichophyton rubrum*, *Microsporum gypseum*, and *Microsporum audouini*. The testing procedure was similar to that described by Goettsch (7), with the exceptions that aqueous solutions of the compounds and a seven-day incubation period were employed. At the end of the incubation period, the minimum zone of inhibition was measured. The zone of inhibition was the distance between the periphery of the paper disk and the colony growth. Compound 21, sodium undecylenate, was used as the control. The results of this antifungal study are given in Table III. Each value in this table represents an average of four zones of inhibition.

DISCUSSION AND SUMMARY

The intermediates 4-chloro-2-butyn-1-ol and 1,4-dichloro-2-butyne were prepared by the procedure described by Bailey and Fujiwara (8). 4-Diethylamino-2-butyn-1-el and the majority of the esters were prepared by the general procedures described by Biel, *et al.* (6). The acetate and propionate esters were prepared by employing an excess of methyl acetate or methyl propionate without the use of the Dean-Stark water separator. The diamines were prepared by reacting an excess of the secondary amine with 1,4-dichloro-2-butyne.

The compounds deleted from Table III were inactive against all of the test organisms, which includes all of the 1,4-bis-N-substituted amino-2-butynes.

TABLE III.—WIDTH OF ZONE OF INHIBITION IN MILLIMETERS (AVERAGE OF FOUR TESTS)

Compound Number	Name	Concn., %	*T. rubrum*	*M. gypseum*	*M. audouini*
2	4-Diethylamino-2-butynyl hexanoate HCl	5.0	6.3	2.0	5.3
		2.5	2.8	0.0	3.0
		1.25	0.0	0.0	0.0
3	4-Diethylamino-2-butynyl octanoate CH_3I	5.0	10.3	3.8	9.5
		2.5	3.5	1.3	8.0
		1.25	0.0	0.0	0.0
4	4-Diethylamino-2-butynyl crotonate HCl	5.0	2.0	0.0	1.0
		2.5	0.0	0.0	1.0
		1.25	0.0	0.0	0.0
5	4-Diethylamino-2-butynyl 10-undecenoate CH_3I	5.0	15.5	13.7	[a]
		2.5	11.8	6.8	30.5
		1.25	11.8	3.3	13.8
6	4-Diethylamino-2-butynyl sorbate HCl	5.0	1.0	30.2	10.5
		2.5	0.0	15.5	9.5
		1.25	0.0	7.2	0.0
7	4-Diethylamino-2-butynyl sorbate CH_3I	5.0	0.0	0.0	0.7
		2.5	0.0	0.0	0.0
		1.25	0.0	0.0	0.0
8	4-Diethylamino-2-butynyl benzoate HCl	5.0	13.5	9.5	7.3
		2.5	6.5	0.0	1.5
		1.25	0.0	0.0	0.0
10	4-Diethylamino-2-butynyl *o*-toluate HCl	5.0	[a]	31.5	30.5
		2.5	36.0	27.8	28.3
		1.25	14.0	17.3	21.5
11	4-Diethylamino-2-butynyl *o*-toluate CH_3I	5.0	0.5	0.0	1.0
		2.5	0.0	0.0	0.0
		1.25	0.0	0.0	0.0
12	4-Diethylamino-2-butynyl *m*-toluate HCl	5.0	9.5	22.7	26.2
		2.5	0.0	11.7	17.5
		1.25	0.0	6.5	12.5
13	4-Diethylamino-2-butynyl *m*-Toluate CH_3I	5.0	0.0	0.0	4.3
		2.5	0.0	0.0	0.5
		1.25	0.0	0.0	0.0
14	4-Diethylamino-2-butynyl *p*-toluate HCl	5.0	23.0	19.5	23.8
		2.5	17.5	7.5	15.0
		1.25	0.0	4.0	12.5
15	4-Diethylamino-2-butynyl *p*-nitrobenzoate HCl	5.0	16.5	8.8	22.0
		2.5	13.0	2.8	10.0
		1.25	7.5	0.0	0.8
21	Sodium undecylenate	5.0	25.0	18.0	37.5
		2.5	25.0	13.5	33.8
		1.25	20.3	6.0	30.0

[a] Complete inhibition.

It was interesting to note that the aminoesters in which both the hydrochloride and methiodide salts were prepared, the hydrochlorides exhibited greater activity than their respective methiodide salts. This correlation was very evident in the benzoate, *p*-nitrobenzoate, and the *o*- and *m*-toluate esters, where their methiodide salts showed little or no activity, while the hydrochloride salts were all of reasonably good antifungal activity. This may be due to the fact that the hydrochloride salts possess a higher degree of water solubility than the methiodides, and thereby diffuse more rapidly across the agar plate surface which was covered with an aqueous spore suspension of the particular organism.

The only ester methiodide showing relatively good activity against all three organisms was the undecenoate ester methiodide. The carboxylic acid portion of the ester was probably an influential factor in this case.

In general, the ester hydrochlorides exhibited activity in the following order: aromatic > alkenyl > alkyl. In this classification, 4-diethylamino-2-butynyl *o*-toluate hydrochloride was superior to the others. It was also superior to sodium undecylenate against two of the fungi and showed excellent activity against the third. The excellent activity of this ester hydrochloride could be attributed to the steric effect produced by the *o*-methyl group, thereby making the ester less vulnerable to hydrolysis and allowing a greater percentage of the compound to exert its activity in the form of the intact molecule.

Further antifungal studies and certain pharmacological testing will be performed on a number of these compounds at a later date.

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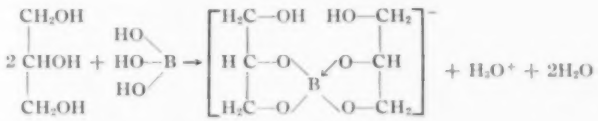
A Solubility Study of the Boric Acid-Glycerin Complex I*

Solubility of Boric Acid in Glycerin Solution at 25°

By JOHN J. SCIARRA and DONALD ELLIOTT

A solubility study of the boric acid-water-glycerin system was made at 25°. The effect of concentration of glycerin upon the solubility of boric acid was noted. Concentrations of glycerin ranging from 0 per cent to 100 per cent, by weight, were utilized in this study. A phase diagram of this three-component system was constructed at 25°. The results of this investigation were compared to the boric acid-water-sorbitol system and a difference was noted between the nature of the boric acid-sorbitol complex and the boric acid-glycerin complex.

GLYCERIN has been used for many years as a solvent for boric acid. This solvent allows the use of approximately 20 per cent of boric acid whereas a saturated solution of boric acid in water contains about 5 per cent of boric acid. Since 1905, the U. S. P. monograph (1) on boric acid has specified the use of glycerin in the chemical assay. The presence of glycerin allows boric acid to be titrated with sodium hydroxide as a monobasic acid (2). Previous investigators have established that a complex compound is formed between glycerin and boric acid although the exact nature of the complex has not been definitely established. This reaction may be represented as follows (3):



If a mixture of five moles each of boric acid and glycerin is heated to drive off all the water formed during the reaction, then a reaction represented by the following equation takes place (4):

$$H_3BO_3 + C_3H_5(OH)_3 \rightarrow C_3H_5BO_3 + 3H_2O$$

This reaction takes place in the preparation of boroglycerin glycerite, N. F., which is used for its antibacterial properties. The former reaction is indicated when boric acid is dissolved in glycerin. Holm (5), Miner and Dalton (6), and Sciarra, Autian, and Foss (7) reported the solubility of boric acid in glycerin. Sorbitol is also known to form a complex compound with boric acid and a solubility study of this system has been reported in a previous publication (8). It is interesting to note that the solubility of boric acid increased as the concentration of sorbitol increased to a maximum solubility of about 19 per cent boric acid in a 70 per cent, by weight, sorbitol solution at 25°.

A saturated solution of boric acid in 70 per cent sorbitol solution can be diluted with water without the precipitation of boric acid. This is not true in the case of a saturated solution of boric acid in glycerin. The addition of water will cause a precipitation of boric acid from such a solution. The formation of a precipitate is one of the problems associated with the use of boroglycerin glycerite when it is diluted with water. Boric acid is formed by the hydrolysis of glyceryl borate. This difference in behavior between solutions of boric acid in glycerin and in sorbitol prompted the following investigation. The solubility of boric acid in various concentrations of glycerin was determined at 25°. From the data, the effect of glycerin upon the solubility of boric acid was noted and the results plotted by means of a phase diagram.

EXPERIMENTAL

Glycerin, U. S. P., containing over 99%, by weight, of $C_3H_5(OH)_3$ was used throughout this study. Solutions of glycerin were prepared by diluting glycerin with varying quantities of distilled water so that concentrations of glycerin were obtained from 0 to 100% glycerin, by weight, in increments of 5%. The specific gravity of each of the solutions, as well as the specific gravity of saturated solutions of boric acid in glycerin, was determined at 25° by the pycnometric method (9). This information enables one to express the solubility of boric acid in several different ways.

The solubility of boric acid in each of the above glycerin solutions was determined by adding a few drops of sodium hydroxide solution until the glycerin solution was slightly pink to phenolphthalein. Then an excess of finely divided, crystalline boric acid, U. S. P., was added to the solvent which had been

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placed into a solubility tube. The tube was then placed into a constant temperature water bath and stirred continuously for twenty-four hours (previous experiments showed that equilibrium had been attained during this period). After allowing the undissolved particles to settle to the bottom of the solubility tube, two 25-ml. portions of the clear, supernatant liquid were removed, placed into tared beakers, and quickly weighed. The amount of boric acid in these solutions was determined by assaying the solutions according to the U. S. P. assay for boric acid (10).

At the same time, a 25-ml. Gay-Lussac specific gravity bottle was filled with the saturated solution of boric acid in the glycerin solvent and the specific gravity determined at 25°.

Temperature remained constant at 25° ± 0.1° by using a Sargent, full visibility, constant temperature water bath.

RESULTS

The results of the above experiments are given in Tables I and II. Table I shows the specific gravity of various glycerin solutions and saturated solutions of boric acid in glycerin solvent at 25° while Table II gives the solubility of boric acid in these same glycerin solvents at 25°. Figures 1 and 2 show these results graphically.

In order to plot a phase diagram of the boric acid-water-glycerin solutions, the percentage composition of each component of this three-component system was calculated and is given in Table III. The phase diagram of this system is shown in Fig. 3.

The results given in Tables I, II, and III represent the average of three determinations for each solvent system.

DISCUSSION

It is significant to note that the glycerin-boric acid complex behaves quite differently from the previously studied sorbitol-boric acid complex. As can be seen from Fig. 1, the curves representing specific gravity of glycerin solvent (A) and specific gravity of a saturated solution of boric acid in the glycerin solvent (B) are separated throughout by essentially a constant. When sorbitol is used as a solvent for boric acid, this difference becomes less and less until finally both curves meet at approximately the specific gravity of a saturated solution of boric acid in 70% sorbitol solution. This seems to indicate that increasing the concentration of glycerin, up to a certain point, does not substantially increase the solubility of boric acid. This can be seen further by observing the essentially straight line from 0% to about 45–50% glycerin solutions in Fig. 2. The slight difference in results is probably due to experimental error. It is interesting to note that the presence of glycerin in these concentrations does not increase the solubility of boric acid. In fact the concentration of boric acid in this range of glycerin solutions is essen-

TABLE I.—SPECIFIC GRAVITY OF BORIC ACID SOLUTIONS IN GLYCERIN AT 25°

Concentration of Glycerin, % w/w	Glycerin Solution	Saturated Solution Boric Acid
0	1.000	1.019
5	1.012	1.031
10	1.024	1.043
15	1.036	1.054
20	1.049	1.065
25	1.062	1.078
30	1.074	1.090
35	1.087	1.102
40	1.100	1.114
45	1.113	1.128
50	1.128	1.142
55	1.140	1.154
60	1.153	1.170
65	1.168	1.181
70	1.181	1.197
75	1.195	1.209
80	1.208	1.222
85	1.219	1.235
90	1.232	1.248
95	1.245	1.262
100	1.260	1.278

TABLE II.—SOLUBILITY OF BORIC ACID IN GLYCERIN SOLUTIONS AT 25°

Concentration of Glycerin, % w/w	Ml. Solvent / Gm. Solute	% by Weight	% by Volume
0	17.58	5.53	5.64
5	17.63	5.33	5.50
10	17.66	5.24	5.46
15	17.67	5.18	5.46
20	17.54	5.21	5.55
25	17.45	5.11	5.51
30	17.19	5.14	5.60
35	16.70	5.22	5.75
40	16.34	5.27	5.87
45	15.49	5.48	6.18
50	14.93	5.61	6.40
55	14.07	5.87	6.77
60	13.06	6.22	7.28
65	11.78	6.77	7.99
70	10.45	7.48	8.96
75	9.00	8.51	10.29
80	7.34	9.89	12.08
85	6.75	10.82	13.36
90	5.19	13.51	16.86
95	4.20	16.04	20.24
100	3.29	19.40	24.79

TABLE III.—COMPOSITION OF BORIC ACID-WATER-GLYCERIN SYSTEM AT 25°

Boric Acid % w/w	Glycerin % w/w	Water % w/w
5.53	0.00	94.47
5.33	4.73	89.94
5.24	9.48	85.28
5.18	14.22	80.60
5.26	18.95	75.79
5.11	23.72	71.17
5.14	28.46	66.40
5.22	33.17	61.61
5.27	37.89	56.84
5.48	42.53	51.99
5.61	47.20	47.19
5.87	51.77	42.36
6.22	56.27	37.51
6.77	60.60	32.63
7.48	64.76	27.76
8.51	68.62	22.87
9.89	72.09	18.02
10.82	75.80	13.38
13.51	77.84	9.65
16.04	79.76	4.20
19.40	80.60	0.00

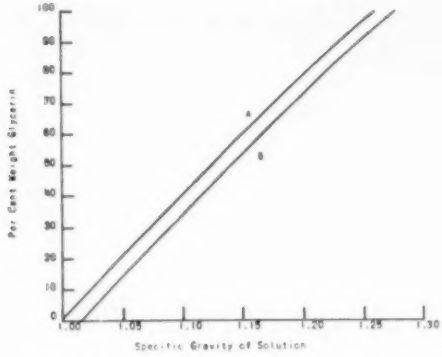

Fig. 1.—Specific gravity of boric acid-glycerin solutions at 25°. *A*, Glycerin solution; *B*, saturated solution of boric acid in glycerin.

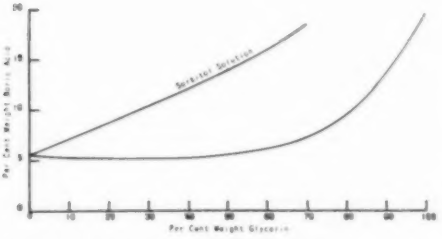

Fig. 2.—Solubility of boric acid in glycerin solutions at 25°.

tially the same concentration of boric acid as found in a saturated solution of boric acid in water. This observation leads one to believe that very little, if any, complex formation, between boric acid and glycerin takes place at this range in concentration of glycerin. Further investigations are indicated to definitely establish this observation. As can be seen from Fig. 2, as the concentration of glycerin is increased beyond 50%, the solubility of boric acid in the solvent increases.

It has previously been established that this increase in solubility is due to the formation of a boric acid-glycerin complex. This complex has a greater solubility than boric acid in the respective solvent system. However, the addition of water to these solutions will cause a precipitation of boric acid, probably due to a change of solvent, resulting in a decrease in glycerin concentration. As has been noted previously, this precipitation does not take place with saturated solutions of boric acid in sorbitol solutions. This indicates the increased stability of the boric acid-sorbitol complex as compared to boric acid-glycerin complexes. Preliminary experiments indicate that propylene glycol reacts similarly to glycerin, which is not surprising when one considers

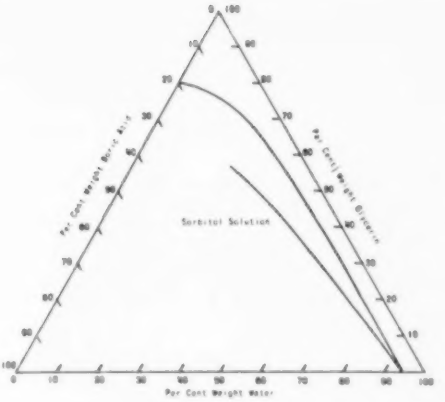

Fig. 3.—Phase diagram of boric acid-water-glycerin system at 25°.

the similarity of these two solvents. The results of this investigation suggest the possible replacement of the boroglycerin glycerite with a similar preparation utilizing sorbitol in place of glycerin, especially when a stock solution of boric acid is indicated. Bacteriological studies may indicate the possible use of this preparation for its antibacterial properties.

The phase diagram of the boric acid-water-glycerin system, Fig. 3, indicates the area of complete miscibility of these three components. This is represented by the area above the curve. As can be seen, the area above the sorbitol curve is greater, indicating a greater area of miscibility for the sorbitol system as compared to the glycerin system. Figure 3 can be used to determine the minimum amount of glycerin necessary to dissolve a given amount of boric acid. This information is essential when one considers problems of formulation.

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Comprehensive Studies on Utah-Grown Medicinal Rhubarb*

By R. L. WORKMAN, Jr., and L. D. HINER

The rhizomes and roots of medicinal rhubarb grown in Utah were subjected to the N. F. X tests for rhubarb. In addition to the official tests, tests for rhaponticin, laxative activity, and organoleptic acceptance were performed. The results of the combined tests indicated that the Utah-grown medicinal rhubarb possessed satisfactory laxative properties, and acceptable taste and odor qualities.

RHUBARB has been known and used as a medicament for over forty-five hundred years. The first written reference to rhubarb was made in the Chinese herbal "Pen-King" which has been attributed to the Emperor Chen-nung (1) and was written about 2700 B.C. Dioscorides referred to *rha* or *rheon* during the first century A.D. *Rha* was the Latin word for the Volga River, and it is possible that the *rha* of that period was rhapontic rhubarb, *Rheum rhaponticum*, from the Black Sea area, although Chinese rhubarb may have reached Europe as early as the first century A.D. (2). The Arabs knew and used rhubarb during the Middle Ages. They were aware that the drug which came from the East via Persia was of Chinese origin and preferred it to the "pontic" variety (3). This preference for Chinese rhubarb has remained until the present time. Indeed, the National Formulary IX imposed a geographic limitation for official rhubarb to China and Tibet (4) and the National Formulary X further restricted this source to China (5).

Because of the source limitation imposed in the N. F., the supply of rhubarb to the United States has always been subject to the political condition of the Far East. This uncertainty of supply has led to investigations of other sources for this medicinal agent. Indian rhubarb (*Rheum emodi* and *Rheum webbianum*) was offered for importation, and after careful study, was admitted to the National Formulary in 1950 as a substitute for Chinese rhubarb. Attempts to grow medicinal rhubarb in the United States have met with very little success. DeRose states that medicinal rhubarb will grow in Massachusetts, Minnesota, and Illinois, but for some reason the medicinal rhubarbs grown in these areas reportedly have an emetic rather than a laxative action (3). Because of the difference in climate between Utah and other areas of the United States in which this drug has been grown, it was decided to investigate the possibility that medicinal rhubarb could grow in Utah that would have the desired laxative action.

The rhubarb used in this investigation was obtained from three sources: Hybrid *Rheum officinale* grown in Utah from seed, Utah-grown *Rheum rhaponticum*, and rhubarb, N. F., obtained from the S. B. Penick Company. The Utah medicinal rhubarb was grown from seed obtained from Dr. Heber W. Youngken of the Massachusetts College of Pharmacy. The seed was planted in the greenhouse in the fall of 1948. The plants obtained from this seed were moved into the field during the spring of 1949. Between 1949 and 1956 these plants were virtually unattended, but still thrived. During the last three growing seasons (1956, 1957, and 1958), the plants were brought under cultivation and, finally, the roots and rhizomes were harvested October 21, 1958. The Utah-grown rhaponticum was harvested at approximately the same time. After harvesting, the roots and rhizomes of both species were washed with water and then carefully peeled. The peeled roots and rhizomes were then placed in a warm room to dry. The official N. F. rhubarb samples were of Chinese origin, and were in granulated and whole commercial forms.

PROCEDURES

Microscopic.—Samples of dried roots of Utah-grown medicinal rhubarb, Utah-grown *Rheum rhaponticum*, and Chinese medicinal rhubarb were placed in 50% ethyl alcohol and left overnight. Normal dehydrating procedures were then used to prepare the samples for embedding in paraffin. Sections made using a rotary microtome were made into permanent mounts and examined with the aid of a research microscope. Samples of the dried roots and rhizomes of all three rhubarbs were ground in a Wiley mill to a number forty powder. They were then examined microscopically using chloral, phloroglucin hydrochloride, glycerine, and iodo-potassium iodide solution as reagents.

Pharmaceutical Preparations.—The preparations used in the organoleptic comparisons and the pharmacological tests were made according to the directions in the official literature. In the case of each

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of the preparations, which included the fluidextract, aromatic tincture, and aromatic syrup, one set of samples was made using official rhubarb and one set using the Utah-grown hybrid.

Tests.—The tests conducted during this investigation consisted of the official tests as listed in N. F. X, some nonofficial but established tests for rhaponticin, and organoleptic comparisons. Official commercial rhubarb was used as the control for the official tests and organoleptic comparisons, while *Rheum rhaponticum* was used as the control for the nonofficial tests for rhaponticin.

Laxative Activity.—Albino mice were used as the test animals in the laxative activity test procedure which was the same as that used for senna and cascara (6, 7, 8). Animals weighing between 20 and 30 Gm. were placed in individual wire cages which were placed upon newsprint. The animals were allowed access to a mixture of powdered Rockland mouse diet and water, mixed in the ratio of 1 Gm. of the laboratory diet to 2 cc. of water, during the entire screening and experimental period. At the end of two hours the newsprint was examined for signs of diarrhea, as shown by sufficient moisture in the stool to stain the underside of the paper. Animals showing signs of diarrhea were discarded and not used in this investigation. The remaining animals were then divided into four groups of 15 animals each.

Two rhubarb fluidextracts were used as experimental drugs: rhubarb fluidextract N. F. and rhubarb fluidextract made with medicinal rhubarb grown in Utah. These fluidextracts were mixed with water in appropriate amounts so that each animal received the desired amount of fluidextract in a volume of 10 cc./Kg. of body weight. This formula resulted in a dosage sufficiently large to permit accurate and ready measurement. All doses were given orally using a hypodermic syringe and a hypodermic needle from which the point had been removed and solder placed on the end to prevent injury to the animal during the administration of the medicament.

Six hours after the administration of the drug, the newsprint beneath the cages was checked for signs of diarrhea. If there were signs of diarrhea the dose of the drug for that animal was considered positive for that particular animal. If there were no signs of diarrhea, the dose of the drug for that particular animal was considered negative. The laxative activities of the two fluidextracts were compared statistically by the method Miller (9) employed to compare the laxative activity of various samples of senna and the cardiac activity of various samples of digitalis.

EXPERIMENTAL

The medicinal rhubarb plants grown in Utah for this investigation at first were thought to be *Rheum officinale* because of information supplied with the seed. Later, however, they would not check out as this species when compared to available keys. Specimens of the leaves and stems with the inflorescence were sent to Dr. Heber W. Youngken. With his cooperation it was determined that the plants used in this investigation were a hybrid of *Rheum officinale* and *Rheum tartaricum* (10).

The roots and rhizomes were examined after they had become completely dry. The dried, unground rhizomes and roots were cylindrical in form, from 6–10 cm. in length and from 1.5–5 cm. in diameter. The outer surface was yellowish brown with patches of brown cork and partially covered with yellow powder. The roots and rhizomes were hard and compact. The fracture was uneven and granular. The newly-fractured surface was pinkish brown in color and, upon standing, a yellow powder formed on this surface. The odor was aromatic and characteristic. The taste was bitter and astringent.

Cross sections of the roots of Utah medicinal rhubarb were compared with cross sections of Chinese rhubarb and rhapontic rhubarb. All three exhibited regular dicotyl structure. The cross sections were devoid of cork cells and cortex because all of the roots were peeled previous to the preparation of the slides. The phloem and cambium were not very pronounced. The xylem was radiate, while the medullary rays were wavy and mostly two cells in width. The tracheae were scattered and in small groups. The xylem consisted of wood parenchyma and contained starch, calcium oxalate rosette aggregate crystals, and some tannin.

The powder of the Utah medicinal rhubarb was of a moderate yellowish-brown color. Microscopic examination of the powder revealed calcium oxalate rosette aggregates from about 40–120 μ in diameter. Starch grains 5–20 μ in diameter, grouped in two's and four's, bunched and single with central cleft hilums were present. There were fragments of vascular rays containing a yellow substance which was soluble in water, insoluble in alcohol, and soluble in ammonia T. S., which turned the substance pink. The vessels were nonlignified, mostly reticulate, with a few elements being spiral.

One per cent potassium hydroxide solution turned both the Utah medicinal rhubarb and the Chinese rhubarb powders a very dark red, which indicated the presence of anthraquinone compounds. The N. F. test for emodin and chrysophanic acid was positive for both rhubarb powders. The Utah medicinal rhubarb contained 35% diluted alcohol-soluble extractive while the official sample contained 39%. Both of these figures are well above the N. F. minimum of 30% diluted alcohol-soluble extractive.

Utah medicinal rhubarb and Chinese rhubarb were tested for rhaponticin while rhapontic rhubarb was used as a control. One-half gram of each powdered rhubarb was shaken with 5 cc. of 2% ammonia solution and allowed to stand in a warm room for fifteen minutes. They were then filtered, and each filtrate was placed on a watch glass. Almost immediately the filtrate from the rhapontic rhubarb had crystals formed in it. At the end of four hours neither the filtrate from the Chinese rhubarb nor the filtrate from the Utah medicinal rhubarb had any signs of crystal formation.

Powdered samples of the three rhubarbs were placed under ultraviolet light to see if rhaponticin could be detected by this means. The rhapontic rhubarb appeared light in color and had a weak purple fluorescence. Both the Utah medicinal rhubarb and the Chinese rhubarb were dark in color, and what little fluorescence was present appeared very dark brown.

Organoleptic tests were performed by a forty-five-member panel consisting of faculty, graduate students, and seniors. The fluidextracts, aromatic tinctures, and aromatic syrups were compared for

color, odor, taste, and preference. Sixty-seven per cent of the panel preferred the fluidextract made with the Utah medicinal rhubarb and one panel member reported that the fluidextract made with the Chinese rhubarb had nauseating qualities. The aromatic tincture made with the Utah medicinal rhubarb was preferred by 67% of the panel and one member of the panel reported nauseating qualities in this aromatic tincture, while another panel member reported these qualities in the aromatic tincture made with the Chinese rhubarb. Sixty-seven per cent of the panel preferred the aromatic syrup made with the Chinese rhubarb. One panel member reported nauseating qualities present in this aromatic syrup.

Two comparison tests were made to determine the laxative activity of the two fluidextracts. The first test compared the laxative activity for both fluidextracts in doses of 0.5 cc./Kg. of body weight and 1 cc./Kg. of body weight. The second comparison was run independent of the first, and the doses employed were 0.25 cc./Kg. of body weight and 0.5 cc./Kg. of body weight. The results obtained in the two experiments were statistically compared by the method outlined by Miller (9). The laxative activity of the rhubarb fluidextract N. F. was arbitrarily assigned a value of one and the fluidextract made from the Utah medicinal rhubarb was compared with it. In the first test the Utah fluidextract had a value of 0.72 with 95% fiducial limits of 0.39 and 1.03, while in the second test this fluidextract had a value of 0.88 with 95% fiducial limits of 0.48 and 1.28. The results were also combined, and a final statistical analysis was made of the combined data obtained with the two fluidextracts. The potency of the N. F. fluidextract was again assigned the value of one and in this analysis the Utah fluidextract had a weighted mean potency of 0.80 with 95% fiducial limits of 0.55 and 1.05. In all three instances the upper 95% fiducial limit overlapped the value of one assigned to the N. F. fluidextract and one must conclude that the two fluidextracts have the same potency.

SUMMARY

1. A hybrid of *Rheum officinale* and *Rheum tartaricum* has been grown in Utah. It grew even when left virtually unattended for a period of six years.

2. Macroscopic and microscopic examination reveals that this hybrid is very similar to commercial samples of Chinese rhubarb and fits the description of rhubarb given in the N. F. X.

3. This hybrid compares favorably with commercial samples of Chinese rhubarb when subjected to the N. F. X tests for rhubarb.

4. Pharmaceutical preparations made with this hybrid are acceptable from an organoleptic standpoint and are relatively free of objectionable qualities.

5. The laxative potency of at least one of its preparations appears to be the same as that of the corresponding official preparation.

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Book Notices

Recent Progress in Oxytocin Research. By B. BERDE. Charles C Thomas, 301 East Lawrence Ave., Springfield, Ill., 1959. ix + 110 pp. 14 x 21.5 cm. Price $4.75.

The extent of accepted knowledge of oxytocin and its definite physiological functions is indicated by the fact that this good review occupies 82 pages. The alphabetically arranged author references (starting with Abel, J. J.) cover 22 pages. Particularly interesting is the second chapter which discusses the relationship between the chemical structure of oxytocin and its biological activity.

Lehrbuch der organischen Chemie. By PAUL KARRER. Georg Thieme Verlag, Herdweg 36, Stuttgart, N. Germany, 1959. xx + 1057 pp. 17.5 x 26 cm. Price DM 60.

This is the thirteenth edition of a classical textbook (in German) an organic chemistry. The book has retained its established style and arrangement, but its text has been considerably revised since the publication of the twelfth edition, which was reviewed in THIS JOURNAL, **43**, 384(1954). Earlier editions have appeared in English translations and it is hoped that this edition also will be translated.

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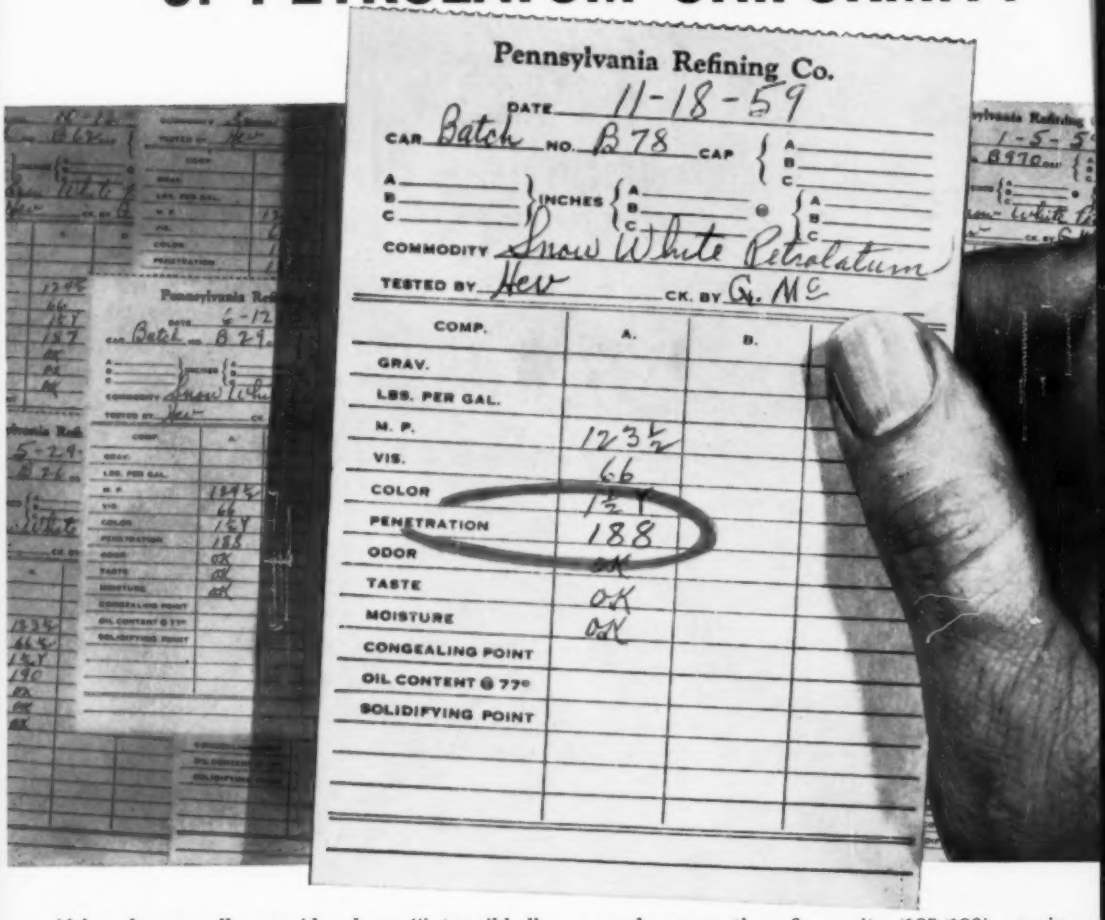

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